

How not to protect databases

A battle is raging between opponents and supporters of a proposed new treaty on database protection. A period of consultation is essential so that information users can properly assess the impact of the treaty.

WITH luck, time will run out at a conference of the World Intellectual Property Organization (WIPO) in Geneva next month. Treaties on the protection of literary and artistic works, and the protection of the rights of performers and producers of phonograms, are on the agenda. But there is also a proposal to protect compilers of databases whose adoption would best be delayed.

The main purpose of the database protection treaty is to prevent anybody who invests significantly in compiling a database from having a return on that investment undermined by someone else copying the data for resale. That practice is all too easy in electronic publishing in particular. Recent court cases have highlighted situations where publishers have spent considerable sums in producing directories, only to find that they cannot protect the product against being copied for profit by others because of the lack of creativity involved in its compilation.

There is, admittedly, more than a whiff of protectionism in the air. In the United States, the information industry is acutely concerned about the effects of a European Directive already adopted in March this year, designed to address this issue but in a way that, some claim, is prejudicial to database suppliers outside Europe. That directive is being implemented in the 15 member states of the European Union and 15 other European countries. US publishers are keen, understandably, to level the playing-field, but are vigorously supporting a treaty proposal that scientists and other database users regard as potentially dangerous and unacceptable.

The word 'database' in the treaty applies to any collection of information that is searchable. As a result, climate data, gene sequences and other scientific data would be included. Frequently, such data have been gathered using public funds. On the face of it, the treaty, and a similar, equally controversial bill before the US Congress, would allow such data to be extracted and sold by a commercial organization which might add little value, yet turn a public good to private gain. Also, the period of protection (15 years is the shortest proposed) could be renewed simply by updating the database. Those possibilities are not in themselves undesirable, but their potential implications for some users appear to be.

What, in particular, of scientists wishing to use the data? There is no mention in the treaty of exemptions for educational and research use, as applies in copyright legislation. True, the WIPO draft treaty would allow the nations that implement it to legislate for exceptions. But the burden of opposition then falls on those traditionally protected (in Anglo-Saxon countries at least) by 'fair use' exemptions from copyright payments, who will have to fight their case in every country. And that against an economic background in which the commercial suppliers themselves, threatened by the communicative promiscuity of digital storage and communication, perceive a need to capture every advantage that they can simply to protect themselves.

Predictions of the effect of the treaty on costs are controversial. Librarians and academics see it as handing a licence to print new money at their expense to commercial suppliers of databases based on government data. The database supplier and the government agency save costs and gain revenue, whereas the public loses a public good and could well be confronted by a monopoly.

One of the few voices endorsing the WIPO treaty is the US Information Industry Association, which represents 550 informa-

tion companies. It claims that the lack of a WIPO treaty undermines its members' confidence in the market and stops them investing, thus reducing choice and quality to US database users. But this glosses too readily over the concerns of what is, after all, an important part of the industry's customer base. In the United States, scientists and other database users have not been adequately consulted. But their worries are justified and more comprehensive debate is needed. That is why discussion of the database protection treaty should be removed from WIPO's December agenda. □

Russian roulette

The fate of Russian science has recently turned from drama to tragedy. Imaginative help from the West is much needed.

GRAND designs to sustain the intellectual vitality of science in the countries of the former Soviet Union have given way to a narrower effort supporting only those parts of the enterprise that Western governments are really concerned about — the science behind Russian weapons of mass destruction. The West's greatest fear is that plutonium or highly enriched uranium could leak to any state, terrorist group or criminal gang. Leaked skills and knowledge are a less direct — if equally potent — concern.

Until recently, the main Russian nuclear weapons laboratories had been sheltered from the disruption that has crippled many other Russian research institutes over the past four years. The weapons laboratories have retained most of their staff, living and working inside large and remote closed cities, far from the distractions which have emptied the corridors of research laboratories in Moscow. This year, however, staff at the laboratories have gone unpaid for months. The recent suicide of Vladimir Nechai, director of Chelyabinsk-70, has underlined the plight of the laboratories.

Western aid for civil science has all but dried up. But Western aid — as well as the military priorities of the Russian government — continues to buttress the weapons laboratories against collapse. The International Science and Technology Center (ISTC) in Moscow, established two years ago by the United States, the European Union and Japan to fund civilian projects proposed by Russian weapons scientists, has properly concentrated on getting grant money straight into researchers' hands. This allows a little money to go a long way. The \$12 million spent on projects at Arzamas, for example, will partially or fully supports 1,200 recipients there for several years. Other US endeavours costing far more — such as the \$1.5-billion Co-operative Threat Reduction programme — are subject to various restrictions to ensure that most of the 'aid' is not actually spent in Russia or its neighbours.

The ISTC is one example of how relatively smaller sums, used as essentially a life support system for Russian scientists, can be equally effective. The financier George Soros realized the importance of this approach in setting up his International Science Foundation. It has also been mirrored by the European aid programme, INTAS. If more funds could be spent in this way, perhaps Russian science could find a way of its tragic situation. □



Russian weapons labs become the top priority for Western funding

Washington. The shock waves that have passed through the international scientific community following the suicide of two prominent Russian science administrators within the space of a few weeks have highlighted the extent to which Western efforts to support science in the former Soviet Union are now focused almost entirely on the activities of the country's nuclear weapons research complex.

Up to 15,000 scientists and engineers are receiving direct cash payments from international sources to reduce the chances of their selling nuclear secrets to criminals or foreign governments. The economic situation in the weapons research laboratories has worsened significantly in the past year, with salaries from Minatom, the agency that runs the complex, being delayed by up to four months.

The sense of crisis was heightened by the suicide last month of Vladimir Nechai, director of the Chelyabinsk-70 weapons research laboratory (see *Nature* 384, 10; 1996), and by the murders and suicide by the director of a computer science institute last week (see below).

But US scientists in regular contact with the Russian weapons laboratories dismiss talk of an impending collapse. The main weapons laboratories at Arzamas-16, 350 km southeast of Moscow, and at Chelyabinsk-70, just east of the Urals, continue to function with staff at around 80 per cent of their 1990 levels. Civilian laboratories, in contrast, have lost at least half their people.

The International Science and Technology Centre (ISTC), which was established in Moscow in 1994 to keep the weapons scientists busy, has already received US\$140

million from the United States, the European Union, Japan and Sweden for the direct support of Russian scientists with expertise in nuclear weapons and other sensitive military technologies.

In contrast to other efforts by Western governments, the ISTC money is distributed directly to each of the Russian scientists engaged in the work.

The system has bypassed both Minatom

George Soros, the billionaire investor, between 1992 and 1995, assistance for civilian science has almost disappeared. Other technical assistance programmes — including \$1.5 billion allocated by the US Congress since 1992 for Cooperative Threat Reduction (often known as Nunn-Lugar money, after the US senators who conceived it) — have been hamstrung by the requirement that the money be spent with US contractors.

Soros is continuing his interest through a \$5-million contribution to the Civilian Research and Development Foundation, matched by \$5 million from the US defence department. But George Brown (Democrat, California), the congressman who initiated the foundation, is bitterly disappointed at the failure of the Clinton administration to support it more generously.

The Department of Energy, which runs the US weapons laboratories, has established a "lab-to-lab" programme in Materials Production Control and Accounting — worth \$15 million in 1995 and \$40 million this year — to control nuclear materials in the Russian weapons complex.

It also has a \$35-million Industrial Partnering Program to encourage technology transfer from Russian laboratories to US industry. The energy department can spend some of that money in Russia, but the bulk will go to US laboratories and contractors.

The US laboratories are allowed to spend a small amount of money on research at their own discretion, and they have been spending some of that on collaboration with Arzamas and Chelyabinsk-70. But the sums are not large, totalling around \$1 million at Los Alamos in New Mexico, for example.

The defence department has also awarded some contract work to Russian scientists. The *Washington Post* revealed last month that, under one such contract, scientists at Arzamas were paid several hundred thousand dollars by the Defense Special Weapons Agency to compile a detailed (but apparently unclassified) history of the Russian nuclear test programme.

Officials at the US State Department say that the total defence department involvement is "hard to pin down". The Central Intelligence Agency does not support work at the Russian laboratories, according to US government officials.

All of this leaves the ISTC as the principal conduit for US support of science in Russia. According to Glenn Schweitzer, the ISTC's first director and author of a new ►

Steven Gitterman

IMAGE UNAVAILABLE FOR COPYRIGHT REASONS

The suicide of Nechai, here with Evgeniy Avrorin, his director of research, caused widespread shock.

and the laboratory directors. But, according to a report released last week by the National Research Council (NRC) in Washington DC, it has been "successful and effective" in meeting its primary goal of redirecting weapons scientists towards non-weapons work, and so diminishing the risk of nuclear proliferation.

The ISTC's dynamism contrasts sharply with the faltering progress of other, more widely publicized efforts to aid civilian science and to stabilize the nuclear weapons complex in the former Soviet Union.

After an infusion of \$100 million from

Institute boss murders five, kills self

Moscow. A new tragedy hit Russia's scientific community last week with the suicide of the head of a research institute in Kazan, shortly after he had shot four of his senior colleagues and his wife.

The affair comes on the heels of the recent suicide of Vladimir Nechai, director of the Chelyabinsk-70 weapons research laboratory.

In the latest incident, 64-year-old Karen Zhamogortsyanyan, who was facing staff efforts to replace him as director of the Kazan branch of the Russian Academy of Sciences' Institute of Informatics Problems, shot dead two deputy

directors, a department head and a senior scientific worker. He then instructed his driver to take him home, picked up his wife and drove her to the city hospital. The driver was sent away after parking the car close to the hospital morgue, and Zhamogortsyanyan shot his wife before killing himself.

As founder of his institute, Zhamogortsyanyan was known to be depressed because, as a result of financial difficulties, the staff were about to elect a new director. The four scientists shot in the director's study were said to be the main supporters of this move. **Carl Levitin**

► book on its origins*, the centre is now reaching some 15,000 of the 60,000 scientists in Russia who possess valuable knowledge of nuclear, biological and chemical weapons and the missiles that deliver them.

At Chelyabinsk-70, he says, there are 5,000 scientists and engineers, and 1,300 of them are being fully or partially supported by \$8 million from ISTC. Some \$12 million has gone to the larger weapons laboratory at Arzamas-16, which has 20,000 technical and non-technical staff.

This intervention has helped to alleviate the latest salary crisis at the weapons laboratories. But according to Schweitzer, the situation there is now just as bad as it was during the previous funding crisis in 1992.

Projects submitted to the ISTC are reviewed on three levels — scientific merit, the level of knowledge of weapons of mass destruction held by the scientists involved, and political acceptability of the proposed research. A proposal to investigate hurricanes by firing missiles into them, for example, was deemed politically unacceptable.

According to Steve Gitomer, a scientist at Los Alamos who arranges the review of US-funded ISTC projects, the quality of proposals varies widely. But he says that some good science is emerging, suitable for publication in refereed journals such as the one he edits — *IEEE Transactions on Plasma Science*.

The NRC report says that the United States should continue to support the ISTC at least until 2003, as it has already pledged to do. It recommends more attention for proposals from scientists with knowledge of biological and chemical weapons.

No-one knows if this effort will ultimately prevent the leakage of knowledge of special weapons from Russia. "I'm somewhat optimistic, but only because it seems that somehow we haven't had any [proliferation] problems so far," says Inta Brikovskis of the NRC staff. "When I see the conditions [in Russia] I wonder how we've been so lucky."

Harley Balzer, a professor at Georgetown University and senior fellow at the Institute of Peace in Washington, who specializes in Russian science issues, worries about the morality of a policy that helps weapons scientists and not others. Balzer thinks that the risk of proliferation, and the justification for supporting the weapons scientists, is greater now than it was in 1992.

Schweitzer, who now works for the NRC but was in Moscow again this week, agrees that Russia and the world face "a very dicey situation" over the next few years. He hopes that the ISTC can help to bridge the gap until the political and economic situation improves and Russia can assert full control over the materials and expertise in its nuclear weapons complex. The alternative is so bad that few are ready even to contemplate it.

Colin Macilwain

*Moscow DMZ: The Story of the International Effort to Convert Russian Weapons Science to Peaceful Purposes (M. E. Sharpe, \$21.95).

Friends and believers bid farewell to Nobel laureate

London. Religion and science blended seamlessly last Monday, 25 November, when, in an apparently unique event for a Nobel science prizewinner, tens of thousands of people gathered in the north Pakistan town of Rabwah to honour the physicist Abdus Salam, who died on 21 November.

Salam, who was 70, had been ill for more than a decade. His body was brought to a mosque in London from his house in Oxford, and flown to Lahore on 23 November. It was then taken by road for burial in Rabwah, near Salam's birthplace. Rabwah is the capital of Pakistan's Ahmadiyya community, a controversial and much-persecuted religious minority of which Salam was a member.

Salam was considered an icon and role model among his fellow Ahmadis, and his grave is expected to become a popular shrine. "When people would visit him, they would kiss his hands," says one prominent community member. "To some, he was like a God."

Salam's passions were divided between theoretical physics, religion, Pakistan and the developing world. Last week, tributes flowed from all four, as well as from the Italian town of Trieste, where Salam was founding director of the International Centre for Theoretical Physics, an institution designed to benefit third world scientists. "This is surely the end of an era," says Luciano Bertocchi, deputy director of the centre.

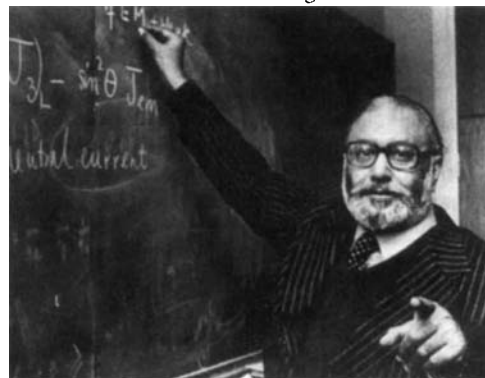
Salam shared the 1979 Nobel physics prize with Sheldon Glashow and Steven Weinberg for unifying electromagnetism and the weak nuclear force. Tom Kibble, professor in the theoretical physics department at Imperial College, London, places Salam among "the first rank of physicists, if you exclude Einstein and Dirac". Kibble joined this department in 1959, two years after it was established by Salam.

Salam remains the only scientist from a Muslim country to have won a Nobel prize. To him, science and faith were inseparable, and he would frequently urge religious leaders to become knowledgeable about science. In a sermon broadcast by satellite on 22 November, Mirza Tahir Ahmad, the head of the Ahmadiyya movement, recalled a discussion in which the Nobel laureate tried to convince Tahir that the speed of light cannot be exceeded. "I told Salam I found this hard to believe as nothing is impossible for God. But he wouldn't have it and drew lots of circles and complicated equations to try and change my mind."

But Salam also suffered for his beliefs in Pakistan, where he was branded an apostate. Followers of Salam's Ahmadiyya faith were

declared 'beyond the pale of Islam' by an international panel of Muslim jurists in 1974. Ahmadis differ from Muslims in believing that the second coming of Christ happened in India nearly a century ago.

Salam felt compelled to resign his post as Pakistan's chief scientific adviser in protest following a wave of anti-Ahmadiyya demonstrations. Later governments have



Salam: loyal Pakistani despite falling out of favour.

shied away from mentioning him too often in public to avoid any potential public and political backlash.

In anticipation of potential crowd trouble — which did not develop — security was tight at Salam's funeral. Unmarked police cars accompanied the procession, and plainclothes members of Pakistan's security forces mingled with the crowds.

The Pakistan government has always denied closing the door on Salam, or precipitating his leaving the country in 1974. The former president, General Zia ul Haq, rolled out the red carpet when Salam returned for a visit after winning the Nobel prize. Tributes to Salam following his death came from the current president, Farooq Leghari, and the newly-installed caretaker prime minister, Malik Meraj Khalid.

Salam never publicly criticized the government and insisted on retaining Pakistani citizenship. This continued commitment to Pakistan baffled many, including some of his Pakistani colleagues. "I have never been able to understand why he was so dedicated to this country when he was virtually ostracized here, being an Ahmadi," says Pervez Hoodbhoy, professor of physics at the Quaid-i-Azam University in Islamabad.

"Salam was never embittered and never gave up trying to do what he could for this country," Hoodbhoy adds. Salam's family say they have not yet decided what to do with his archive. But plans are being discussed to put a biography on the Internet.

Ehsan Masood

An obituary of Abdus Salam will appear in a future issue.

Gene panel reprieved after public outcry ...

Washington. Harold Varmus, director of the US National Institutes of Health (NIH), last week announced a compromise on the future of NIH's Recombinant DNA Advisory Committee (RAC). The committee's role as a public forum for discussion of issues relating to gene therapy will be preserved, although its responsibility for approving specific protocols will be scrapped.

The proposal, outlined in the *Federal Register* on 22 November, reverses Varmus's earlier call for the abolition of the RAC, whose members include scientists, lawyers, ethicists and consumers (see *Nature* **381**, 635 & **382**, 197; 1996). The committee — reduced in number from 25 to 15 — would meet to discuss protocols that a majority of its members agree are novel enough to justify public discourse. But it would no

longer vote to approve or block protocols.

That responsibility would rest solely with the Food and Drug Administration (FDA). The new procedure would also eliminate the role of Varmus, who currently receives approved protocols from the RAC and gives (or occasionally withholds) his approval before sending them to FDA.

The announcement in the *Federal Register* hints at the outcry that followed Varmus's initial proposal in July to eliminate the RAC, a 22-year-old committee which has provided a public forum for debating issues raised by recombinant DNA technology. The modifications come "in response to public opinion" and the view that the RAC has "historical importance" as a platform for discussion of gene therapy, the proposal says.

"My goal was to expand public discussion

about gene therapy now and in the future, avoid duplication with the FDA, and maintain the public database," says Varmus. "This proposal accomplishes those goals and responds to public interest in retaining NIH oversight of human gene therapy".

The NIH received 71 communications in response to its initial proposal, published in July; opponents of RAC's elimination outnumbered supporters by two to one. Support came mainly from biotechnology and pharmaceutical firms.

The new proposal, which the RAC itself will respond to at a meeting on 9 December, departs significantly from the earlier one, which would have replaced the RAC with a smaller committee charged primarily with convening gene-therapy policy conferences on novel topics.

Abbey Meyers, president of the National Association for Rare Disorders and a member of RAC, calls the new proposal "wonderful". She says that Varmus has "accomplished his purpose" — to avoid adding NIH's stamp of approval to mediocre proposals approved by the RAC, which is charged with judging not scientific quality but ethics.

In contrast, Paul Berg, the biochemist at Stanford University whose concerns in the 1970s were largely responsible for the creation of the RAC, called the revised proposal "an intermediate step" towards abolition. Berg believes that the RAC has outlived its usefulness.

But W. French Anderson, the molecular geneticist at the University of Southern California who pioneered *ex vivo* human gene therapy in 1990, calls the new proposal an "excellent compromise". He wants the panel to continue to vote on the protocols it reviews, because, even though such votes lack any authority, they will have influence.

Another RAC member, Robertson Parkman, a paediatric immunologist at the Children's Hospital in Los Angeles, says that whether the restructured committee takes votes is "irrelevant". The main need, he says, is to have "the pros and cons discussed in a public fashion". But he points out that the current proposal does not specify the threshold of novelty at which a protocol would trigger a majority of RAC members to call for a public discussion.

The revised proposal — provisional until Varmus issues a final version after the next RAC meeting — retains the proposed gene-therapy policy conferences. These will be convened to coincide with RAC meetings and co-chaired by RAC members. The conferences are intended to deal with novel and controversial issues such as *in utero* gene therapy and germ line therapy. Comments on the revised proposal should be sent to the NIH Office of Recombinant DNA Activities by 2 December.

Meredith Wadman

...as concern grows over screening

San Francisco. Companies marketing the first broad-based genetic tests available in the United States are raising unnecessary alarm and promising impossibilities, according to geneticists, bioethicists and others who took part in a forum on genetic testing last week.

"One of my fears is that we will commit so many egregious errors early on that the American public will decide that they do not want to have anything to do with this technology," said Francis Collins, director of the National Center for Human Genome Research at the National Institutes of Health (NIH). "We don't need a genetic thalidomide."

The forum was organized in San Francisco by the Stanford University Program in Genomic, Ethics, and Society. The centrepiece of the meeting was the presentation of guidelines developed by a 53-member panel convened by Stanford to study the introduction of genetic tests for mutations in *BRCA1* and *BRCA2*, which confer susceptibility to breast cancer.

In a keynote address, Collins criticized advertising by the Genetics and IVF Institute, based in Virginia, that appealed to Jewish women, regardless of family history, to be tested for the *185delAG* deletion. This mutation in *BRCA1* is found in about 1 per cent of women of Eastern European Jewish ancestry.

Neil Holtzman, chair of a joint NIH/Department of Energy Task Force on Genetic Testing, chastized companies for their advertising of genetic tests for breast cancer susceptibility. Among other problems, he claimed that Myriad Genetics, of Salt Lake City, Utah, and IVF

overstate the risk of breast cancer among women with the *BRCA1* and 2 mutations by using data from the most dramatically affected families.

Participants at the meeting expressed alarm at statements made in a presentation by Mark Skolnick, executive vice-president of research for Myriad, about the company's decision to offer full sequence testing for mutations in *BRCA1* and 2, at a price of \$2,400.

Skolnick argued that claims by the Stanford group about the uncertainty of the tests' benefits were irresponsible and misleading. The Stanford guidelines say that there are no known methods for preventing breast or ovarian cancer that would particularly help women with the mutations; Skolnick said prophylactic surgery was an effective preventive measure that could be taken by women found to have a mutation in *BRCA1* or 2.

The Stanford guidelines advise most women not to take the test, unless they have a strong family history of breast or ovarian cancer. They call on the Food and Drug Administration (FDA) to regulate all genetic tests and their marketing. The agency should develop new rules that require evidence of safety and efficacy — not only in a medical sense, but also psychologically and socially — before genetic tests enter the market, said Hank Greely, professor of law at Stanford and chair of the panel.

Sally Lehrman

• Myriad Genetics announced on Monday that it had dropped plans to proceed with a public stock offering, on the grounds that "conditions are not favorable to going forward at this time".

Regions picked as biotech 'winners'

Munich. Three regions in Germany emerged last week as winners of a well-orchestrated competition called BioRegio, run by the federal research ministry and intended to promote collaboration in biotechnology between academics and industry.

Jürgen Rüttgers, the research minister, named Munich, the Rhine-Neckar Triangle (including Heidelberg, Ludwigshafen and Mannheim) and Rhineland (including Cologne, Düsseldorf and Aachen) as Germany's 'biotechnology model regions' (see map). Seventeen regions had applied. They were asked to provide realistic and innovative ideas for the development of biotechnology in their regions.

Munich's winning concept focuses on the development of genome-based diagnostic and therapeutic products. Its new venture-capital company, Bayern Kapital, has already attracted DM30 million (US\$20 million) of private investment. Rhineland's concept focuses on both biomedical and plant-breeding products, and its two new venture capital companies have attracted DM110 million.

In the Rhine-Neckar Triangle, banks and large pharmaceutical companies such as Boehringer and BASF have jointly founded the Biotechnologiezentrum (Biotechnology Centre) in Heidelberg to support scientists who want to start businesses. A third of the region's suggested projects involve the Heidelberg-based national German Cancer Research Centre. They include the develop-

ment of vector systems for gene therapy and basic research in areas such as AIDS.

When the competition was launched a year ago, academics, industrialists and politicians in Germany rose to the challenge with unprecedented fervour, despite the vagueness of the promised prize. In fact, the prize costs the ministry nothing — or at least only the effort of rearranging its existing budget. The ministry has separated DM30 million per year from its budget for biotechnology over the next five years, and the three winning regions will have 'preferential access' to this money.

The ministry did, however, spend DM1.7 million supporting the expensive application procedures for the competition, with each

region that applied receiving half its costs, up to DM100,000. Most regions worked with international business consultancies such as Ernst and Young (Rhine-Neckar Triangle) and Prognos (Munich). And though the tangible prize is small, the prestige of the title should prove a strong attraction to future investors.

The winning regions clearly reflect the current concentration of biotechnological expertise in south and west Germany. Nevertheless, Jena, the east German centre for optics research, was given a 'special merit' by the jury for its plans to create from scratch an environment for biotechnological research.

Peter Radunski, research minister for Berlin, which, with east Germany's Brandenburg, was placed fourth in the competition, noted with regret that "the jury of the BioRegio competition made conventional decisions".

Losers in the competition have clearly benefited from the contacts made when developing their applications. But Rüttgers' optimistic claim that there are no real losers rings a little hollow. The earmarking of some of the ministry's research funds for competition winners reduces the size of the remaining pot for which all German biotechnologists can apply. "This will clearly disadvantage other regions," says Harald zur Hausen, director of the German National Cancer Centre (DKFZ).

Quirin Schiermeier & Alison Abbott



European convention allows use of human embryos

Munich. An international convention permitting the use of human embryos for research — but forbidding their creation for such purposes — was adopted last week by the Council of Europe, an intergovernmental body to which 40 states belong.

The Convention on Human Rights and Biomedicine, which sets out a legal framework in which genetic research can be carried out on humans, also forbids germ-line therapy.

But Germany, Poland and Belgium abstained from voting for the convention because it does not impose a total ban on embryo research, and because it also allows research in certain circumstances to be carried out on humans unable to give consent, such as children, the mentally handicapped and patients in a coma.

Failure to reach a consensus on these issues was largely responsible for the rejection of the first draft of the convention by the council's parliamentary assembly two years ago (see *Nature* 371, 643; 1994).

The revised document jettisons some of the original controversial clauses, such as that forbidding research on embryos more

than 14 days old — which some argued gave implicit approval to research on embryos before 14 days. The convention now merely states that "where the law allows research on embryos *in vitro*, it shall ensure adequate protection of the embryo". A more detailed agreement on embryo research, which will have to be approved by the council's parliamentary assembly and council of ministers, is now being discussed.

The convention forbids the creation of human embryos for research. But Britain, the only Council of Europe member state with a law specifically allowing the creation of embryos for research, will probably opt out of this provision when the convention is ratified by the UK parliament. Any country is entitled to opt out of individual clauses if its existing legislation contradicts them.

A spokeswoman for the UK Human Fertilization and Embryo Authority says that in practice most of the 20 research projects using human embryos that the authority has licensed this year use spare embryos arising from *in-vitro* fertilization.

The revised convention also provides greater protection than the earlier draft to

individuals used for research who are unable to give their consent. This is a particularly sensitive issue in Germany with its history of Nazi abuse of concentration-camp victims (see *Nature* 384, 5; 1996).

In general, says the convention, any medical intervention may only be carried out on an individual who has given free and informed consent. When an individual is not able to give such consent, the intervention must be for his or her direct benefit, and such a person must be allowed to take part in the authorization procedure "as far as possible".

The convention makes broad statements about genetics, forbidding discrimination on grounds of genetic inheritance, for example, and allowing genetic tests to be carried out only for health purposes or research related to health, and only after appropriate genetic counselling. In particular, it forbids germ therapy and the use of genetic techniques to determine the sex of a fetus, except to avoid serious sex-related disease.

The convention must now be ratified by each member state individually. A.A

US science lobby intensifies attack on database pact . . .

Washington. US scientific leaders have stepped up their attacks on a proposed treaty on ownership of computer databases drawn up by the World Intellectual Property Organisation (WIPO). The scientists argue that it could undermine the activities of researchers in disciplines ranging from climatology to human genetics.

The biotechnology industry has joined professional organizations representing most US physicists and biologists in condemning the draft of the proposed treaty. It is one of three draft treaties on intellectual property that will be discussed in three weeks of negotiations starting next Monday (2 December) in Geneva.

The critics believe that the US Patent and Trademark Office (PTO) has endorsed a draft that favours the commercial compilers of databases, and has left the views of scientists and other database users out in the cold (see *Nature* **383**, 653; 1996).

The National Research Council (NRC), the operating arm of the National Academies of Science and Engineering, last week took the unusual step of releasing an early draft of an assessment of the draft treaty by its committee on scientific data exchange.

The review condemns the WIPO draft as "overly protectionist", and calls on the White House Office of Science and Technology Policy (OSTP) to slow work on it "to a rational pace", to consult scientists properly and to ensure a "fair use" provision

that guarantees free access to information for research purposes.

Science lobbyists in Washington are criticizing OSTP for failing to spot problems with the draft earlier in the year. The text was prepared by the PTO in the US Department of Commerce and has the support of the US government. But OSTP officials argue that they addressed the issue promptly, and that scientists' concerns will be taken into account at the Geneva negotiations.

At a meeting organized by the NRC last week, David Schmickel of the Biotechnology Industry Organisation said that his industry was "united in opposition to this treaty, because of the uncertainty it would cause". Schmickel said the draft treaty defined databases too broadly, would obstruct public access to gene sequence data and fragment sources of it and would lock data into perpetual protection by allowing compilers to keep rights to databases for 15 years after updating them.

Kenneth Berns, president of the American Society for Microbiology, has written to the PTO expressing "great concern" about what he terms the "arbitrary, nonrepresentative and inappropriate position" being taken by the US government to Geneva. He wants the database treaty to be taken out of the talks. The American Physical Society and other groups, such as the Association of American Universities, are also mobilizing on the issue.

Colin Macilwain

Transatlantic merger creates potent hybrid in bioscience research

Oxford. Chiroscience, one of Britain's leading entrepreneurial bioscience companies, has signalled its intention to become a major force in drug discovery by merging with the US biotechnology company Darwin Molecular. Darwin, based in Seattle, Washington state, counts Bill Gates, the founder of Microsoft, as one of its backers.

The merger, valued at US\$120 million, is the largest deal initiated by a UK bioscience company and involving a US company, and may become a model for others. British bioscience companies currently enjoy stronger valuations than their US counterparts, and investors in private US biotech companies may welcome the opportunity of trading their stock for shares in British companies.

Chiroscience has focused on applied research and development using chiral and other synthetic chemistry techniques to yield drug candidates, as well as having one of the most impressive research pipelines in an entrepreneurial bioscience company. It has already seen one of its products move into the marketplace, and 11 other programmes are under development.

But Chiroscience officials believe that the company's value can be significantly enhanced with the introduction of new development programmes, and a broadening and strengthening of its technology base. "Darwin is expected to help fulfil both criteria," says John Padfield, Chiroscience's chief executive officer. "The acquisition represents a significant step in potentially increasing the efficiency of development programmes through the introduction of new technologies more rapidly than would have been achieved through in-house development."

Since its foundation in 1991, Darwin has been focusing on discovering and developing diagnostic and therapeutic products for autoimmune diseases, such as psoriasis and rheumatoid arthritis, and for certain cancers. This has involved the integrated application of gene discovery, molecular biology, combinatorial chemistry and bioinformatics. Scientists at the company recently identified key genes involved in two genetic disorders, Werner's syndrome, which accelerates the ageing process, and early onset of Alzheimer's disease.

The new transatlantic company will have nearly 300 employees, about 80 per cent of them in research and development. The deal will not drain Chiroscience's coffers as Darwin shareholders are being offered Chiroscience stock worth some \$120 million rather than cash. After the merger, Darwin shareholders will own about 20 per cent of the combined company.

Mike Ward

...as opposition makes delay likely

Paris. The proposed international treaty on the legal protection of databases looks increasingly unlikely to be adopted next month as planned, following growing opposition from the research and education communities, particularly in the United States.

The draft treaty is scheduled to be adopted at a Diplomatic Conference of the World Intellectual Property Organisation (WIPO) which begins next week in Geneva. But one WIPO official says that a "large effort" will be needed if agreement is to be reached. "The question is whether the treaty is right for adoption," he says.

The draft treaty has attracted broad opposition from scientific organizations in the United States, as well as from the International Congress of Scientific Unions (see above). They warn that its provisions could have a damaging effect on research and teaching. But the WIPO official attributes much of the strong US

opposition to the fact that it has not yet introduced corresponding domestic legislation, resulting in concern that the issues raised by the treaty have not been properly discussed.

The treaty is based largely on a European Union (EU) directive on the legal protection of databases, adopted earlier this year following eight years of broad consultation, and must be implemented by all member states by 1998. One official points out that the unanimous adoption of the directive — no mean feat in Europe — "demonstrates that it is rather balanced".

But the WIPO treaty differs from the EU directive in that its wording is more vague and ambiguous. Advocates of the treaty say this is unavoidable in international treaties, if consensus is to be reached among the many signatories, and that it is up to individual countries to clarify the text when they implement it in domestic legislation.

Declan Butler

IMAGE
UNAVAILABLE
FOR COPYRIGHT
REASONS

Associated Press

Aeronomics institute leads protests against closures

Constantinescu: from minerals to presidency.

Romanian scientist elected president

London. Romania made history last week by electing a 57-year-old research scientist and chancellor of the University of Bucharest as the first non-Communist president since the revolution of 1989. Emil Constantinescu, a mineralogist, defeated incumbent president Ion Iliescu by a comfortable margin of 10 per cent to secure an overall majority in parliament for his Conventia Democrata party.

Romania's 1989 revolution had left a Communist elite in power. Iliescu was a one-time heir-apparent to the dictator Nicolae Ceausescu, and his regime increasingly became the focus of public unrest.

From relative obscurity as a university chancellor, Constantinescu's public profile began to rise when he joined student protests against the Iliescu regime's alleged disregard of academic freedom. Earlier, mobs had stormed the university buildings and destroyed most of Constantinescu's collection of microscope mineral slides.

Opinion polls suggest that in the presidential elections he attracted a large proportion of the under-35 voters, who were attracted by his clean and untainted image. "There are no dark corners in my life, so I cannot be blackmailed," was one of his campaign slogans.

Before entering politics, Constantinescu — whose first degree is in law — was a prolific scientist and published mineralogy papers in Romanian, French and English journals. He concentrated on research under the Ceausescu regime, as a freeze on academic promotions ruled out a move into university management. He was appointed vice-chancellor soon after Ceausescu's fall, and took over as chancellor in 1992 (see *Nature* 372, 606; 1994).

Constantinescu has a tough job ahead of him. Half a century of Communism has crippled Romania's economy, once prosperous on the back of oil, gas and mineral wealth. He is also unfortunate in that most of his competitive neighbouring new Eastern European democracies have a head start in their transition to a free-market economy.

Constantin Roman

Munich. Scientists at the Max Planck Institute for Aeronomics in Lindau, Germany, took to the streets earlier this month as part of a vigorous campaign to save their institute from closure. But the campaign, which includes soliciting support from scientific colleagues around the world, appears so far to have made little impact.

Last week the senate of the Max Planck Society endorsed the proposal of its president, Hubert Markl, that staff numbers be reduced by 512 in west Germany before the end of the decade by closing four institutes, including the Lindau institute, and at least ten departments (see *Nature* 383, 566; 1996).

The harsh measures have been prompted by an across-the-board cut in public-sector jobs imposed by the federal government, combined with a political decision within the society to develop its institutes in east Germany to western levels.

All four institutes earmarked for closure have launched protests, but none has been more forceful than that of the aeronomics institute. It has set up an Internet home page and has sent more than 700 letters to colleagues asking for support. More than 130 have already responded by writing to Markl asking for a reprieve, or at least for protection of the institute's current commitments to planetary science.

A demonstration held jointly with the Max Planck Institute for History — also under threat of closure — in neighbouring Göttingen attracted more than 500 people.

Young scientists at the aeronomics institute have been actively involved in the

campaign to keep it open. One reason is that their future careers depend on Germany not pulling out of planetary science, according to Norbert Krupp, a 33-year old physicist who is helping to develop a microdetector for an instrument aboard Cassini, the US-European mission to Saturn due for launch next year.

There is no other large institute in Germany specializing in planetary science, and the 30 or so young scientists at Lindau have already become too specialized to change track easily, Krupp says.

Markl has repeatedly promised that commitments to international projects such as the European Space Agency's cometary mission Rosetta and Cassini will be fulfilled. He hopes that the projects can be transferred to DLR, Germany's national research centre for space research.

If DLR chooses not to cooperate — which seems likely, given the pressure on its own budget — the Max Planck Society will find a way to support the projects itself, insists Markl. But, asks Krupp, who will be around to analyse the data from instruments developed in Germany, if German planetary science is allowed to die before the missions reach their target planets?

Markl says he plans to continue to consult widely before his final proposal goes through its formal first reading at the next MPS senate meeting in March. He says that he hopes a final decision will be reached within the following few months, so as not to stretch out the "cruel waiting time" for the scientists involved.

Alison Abbott

Dual role for Australian research chief

Sydney. The Australian government has appointed John Stocker as both its chief scientist and the chairman of the Australian Science, Technology and Engineering Council (ASTEC).

Stocker was director of research for the pharmaceutical company Hoffman-La Roche in Basel, Switzerland, before returning to Australia to serve as chief executive of the Commonwealth Scientific and Industrial Research Organisation from 1990 to 1995.

His appointment has been widely welcomed, but there is less enthusiasm about the role he has been asked to fill. Stocker will be responsible in both posts to the minister for science and technology, Peter McGauran, who is not a member of the cabinet. Under the former Labor government, both his predecessors reported to the prime minister, although Stocker says he retains an "advisory line" to the premier.

Further concern that the status of the

chief scientist post is being downgraded centres on the fact that it will be only a part-time position. Stocker will spend one day a week in each post, while continuing as a company director, a consultant to technology-based industry and a professorial fellow in the University of Melbourne.

Joe Baker, president of the Federation of Australian Scientific and Technological Societies, argues that the chief scientist's position is "too big to be handled in one day a week". Labor's science spokesman, Martyn Evans, says the changes "confirm that the government has downgraded science".

But Stocker maintains that his experience and his close connections with industry will benefit the government's stated intention of improving links between science and the economy. And McGauran rejects claims that the role is diminished by Stocker working one-fifth of the combined time of his predecessors.

Peter Pockley

Trade war looms over gene-altered foods

Paris. The European Parliament, the parliamentary assembly of the European Union (EU), has adopted a resolution calling for genetically modified products to be labelled as such and sold separately from non-modified products.

The move represents a boost to an international boycott of genetically modified soya beans and maize by more than 300 consumer, health, trade and farming organizations. But it sets the parliament on a collision course with European governments, most of which favour labelling products only where they are substantially modified.

It also raises the prospect of a trade war with the United States, as any move to ban the import of foodstuffs that cannot be labelled in the way the parliament is calling for could be declared illegal under the rules of the World Trade Organization (WTO), which has taken on responsibility for the General Agreement on Tariffs and Trade (GATT). The rules allow exclusion only on strictly scientific grounds.

The parliament is still a long way from having its demands incorporated into EU legislation. Labelling is included in the European Commission's draft legislation on novel foods, and the parliament's stance is at odds with that of the council of ministers, which represents the EU member states. At present, only Austria, Germany, Denmark and Sweden support full labelling.

A political stalemate is therefore highly likely, as the parliament and the council need to agree on the legislation if it is to be adopted.

But, whatever the outcome, some observers argue that mounting public support for labelling represents a direct challenge to both international trade law and the practices of regulatory authorities. Labelling brings with it the right to choose what sort of foods to buy, not only on safety grounds but also on others such as opposition in principle to genetically modified organisms.

Regulatory authorities, under pressure from biotechnology and food companies, have until now refused systematic labelling unless the product is "substantially modified". They argue that labelling would tend to stigmatize biotechnology, while providing little useful information to consumers.

The current controversy centres on the fact that this year's exports of US soya beans to Europe contain a small proportion of Monsanto's genetically modified product, leaving importers with the choice of accepting the mixed shipments or refusing all US imports of soya beans.

The European Commission, which has already approved the marketing of the beans, will decide next month whether to approve Ciba's modified maize. If it does not, it will similarly need to ban all US maize

imports — which again contain modified material — and risk triggering a trade war.

The possibility that the commission will reject Ciba's maize (which has been approved in the United States, Canada and Japan) cannot be discounted, according to observers. The UK Advisory Committee on Novel Foods and Processes is concerned about the safety of a marker gene in the maize that confers resistance to antibiotics. Given that this dissent exists, it would not be abnormal for "the doubt to prevail", says Axel Kahn,



head of France's Commission du Génie Moléculaire (which has approved the maize). "If the European Commission opposed the product it would not shock me at all."

The commission is under pressure following the public opposition to imports of modified soya beans. Many food retailers, such as the UK Iceland Group, are supporting the consumer campaign for labelling, while some importers have refused all US soya bean exports — Unilever Germany, which usually accounts for 7.5 per cent of all EU imports of US soya beans, has done so, for example. Nestlé last week also bowed to consumer pressure and said it would label genetically modified produce "where reasonable and practicable".

According to Jeremy Rifkin, director of the Foundation for Economic Trends, a US lobby group, and long-time critic of genetic engineering, the fact that grain dealers are taking action beyond that required by international trade law shows that the law is out of step with the needs of consumers and the retailers wanting to satisfy their needs.

"This issue of soya and corn labelling is the lightning rod issue that will test our trade agreements," says Rifkin. "If trade agreements come up against consumer sovereignty, they are going to have to give way," he says, adding that such agreements need to be more open to public health concerns.

Others, such as Marie-Angèle Hermitte, an academic legal expert at the French Centre National de la Recherche Scientifique, argue similarly that the focus of trade agreements on the principle of free trade places excessive responsibility on countries to

prove that a risk exists in cases where they seek to control the movement of goods on public health and safety grounds.

In practice, agreements reached in the most recent round of GATT negotiations have greatly reduced the scope for individual countries to ban imports on public health grounds. The changes were felt necessary to curb the misuse of such arguments as a pretext for protectionist measures.

Under the rules of the WTO, countries may not refuse imports or demand that they be labelled, for example, unless they can show that they fail to meet agreed standards where these exist, or otherwise present firm scientific evidence of a risk.

If the EU were to bow to consumer pressure and label genetically modified products, the United States could argue that this was a disguised barrier to trade "in that the labels could be seen as a means of scaring people into buying European alternatives", says Phil Evans from the UK Consumers' Association, which supports full labelling.

WTO regulations also state that countries cannot discriminate between identical products by labelling them differently. The organization has ruled, for example, that tuna caught in a way that kills dolphins cannot be labelled differently from that caught in a way that does not, according to Sabrina Shaw, an economic adviser in the WTO's trade and environment division.

WTO has not specifically considered genetically modified products, however. Shaw adds that it is unclear, for example, whether this precedent would apply if genetic changes were detectable in the final modified product.

Shaw points out that trade laws can favour public health by obliging countries to harmonize their domestic standards with international standards. WTO rules also allow a country to invoke higher standards than the international norm, but it must provide scientific evidence in support of its case, and also must apply the higher standards to its domestic industry as well as to imports.

Some are confident that, even if the commission were to reject Ciba's maize, it could still avoid a showdown with the United States. "If we had scientific advice which would give us some arguments of a risk, we would be in a good position," says one official from the European Commission, adding that much would depend on whether the United States would be simply "unhappy" or complain to the WTO.

Stephen Smith, managing director of Ciba Seeds UK, is philosophical about the prospects that the commission might reject its application. "Once the commission makes its decision we will take stock and see where we will go," he says. "If the commission refused, it would be a setback but it would be surmountable." **Declan Butler**

Genetic testing resolves royal riddle of German foundling

Munich. Geneticists in Birmingham and Munich have solved the long-running mystery surrounding the identity of the nineteenth century foundling Kaspar Hauser: he was not, as has been claimed, a noble heir. Hauser was found in Nuremberg in 1828, having apparently spent the first 16 years of his life held captive in a hole dug in the forest. Many believed him to be the son of the Duke of Baden, who had been kidnapped shortly after birth to allow the children of the Duke's second wife to inherit.

Over two thousand books have been written about the affair, but only now have scientists proved conclusively that he was, in fact, not the royal heir. The town of Aurbach, near which he died in 1833, last year commissioned a genetic analysis of Hauser's blood to put the long-debated matter finally to rest. Samples from a blood stain on the suit Hauser was wearing when he died yielded sufficient material to compare its DNA with that contained in blood of two direct relatives of Hauser's supposed natural mother. No match was found. However, the circumstances of his violent death remain a mystery. □

Reef fishing research cleared

Canberra. The world's largest experiment on the effects of fishing on coral reefs has narrowly survived a challenge in the Australian parliament by a margin of two votes, following the claims of critics that the scientists were following the dictates of the commercial industry and would use dynamite and poison in their tests — charges dismissed by the researchers as “disinformation”.

The experiments are planned to take place over five years on the populations of coral trout on 24 of the 3,000 reefs in the Great Barrier Reef off the east coast of north Queensland (see *Nature* 384,

9; 1996). The government's support of necessary zoning changes has been vigorously opposed by the Democrats and Greens in the Senate, while the Labor party had claimed that the Environment Minister, Robert Hill, had bypassed another level of approval required under World Heritage provisions. But the government prevailed when two independent senators refused to join its opponents. □

Congress confirms science chair

Washington. James Sensenbrenner (Republican, Wisconsin) has been confirmed as the next chair of the Science Committee of the US House of Representatives. Sensenbrenner, a 53-year-old lawyer and staunch fiscal conservative whose main interest on the committee hitherto has been the oversight of the National Aeronautics and Space Administration, succeeds Robert Walker (Republican, Pennsylvania), who has retired.

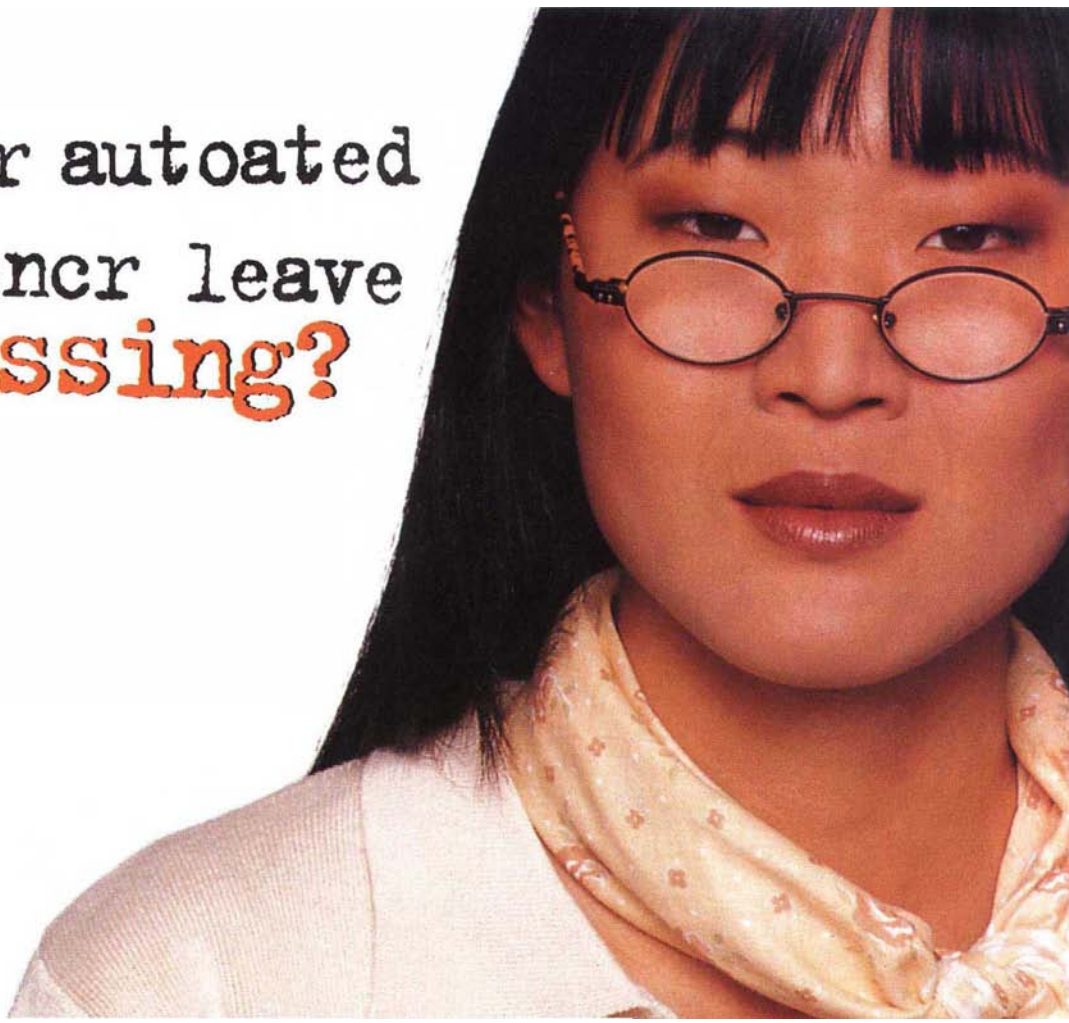
Joseph McDade (Republican, Pennsylvania) will chair the energy and water appropriations subcommittee in the House, which funds the Department of Energy. The chairmanship of the other major House committees responsible for science oversight and funding remain unchanged. □

Top astronomy citations

London. The NASA-Goddard Space Flight Center in Maryland and Britain's Royal Observatories have emerged at the top of league tables for astronomy and astrophysics research. Papers from NASA (the US National Aeronautics and Space Administration) received 2,521 citations between 1993 and 1995, according to an analysis of 1,359 papers by the Philadelphia-based Institute for Scientific Information. This was 339 citations more than the second-placed institution, the Harvard-Smithsonian Centre for Astrophysics. The Space Telescope Science Institute came third.

But Britain's Royal Observatories, which came seventeenth,

Does yur autoated
DNA seqencer leave
u **guessing?**



London. Three UK research councils are to share the £3-million costs of upgrading the Synchrotron Radiation Source (SRS) at Daresbury in Cheshire, a source of intense light used to probe the atomic structure of materials. The upgrade will be paid for by the Biotechnology and Biological Sciences, the Medical, and the Engineering and Physical Sciences Research Councils.

Munich. Germany's Max Planck Society (MPS) is to dip into its private funds to create ten new high-level five-year positions for women over the next three years. Hubert Markl, the president of the MPS, says that, despite acute cash shortages, the very low representation of women among department heads must be corrected as a priority. Women occupy only six of the society's 185 C3 positions — the second-highest rank for scientists — and five of the 225 top-ranking (C4) positions. "In a male-dominated society like science, it is important to consider the problems faced by women very seriously," he says, regretting that the posts will have a relatively small overall impact. □

Washington. Only nine patients — fewer than one in five of those invited — have agreed to participate over the past five months in a long-term follow-up study by the University of Pennsylvania Cancer Center of patients with *BRCA1* and *BRCA2* mutations, which predispose to breast and ovarian cancer. Many prospective participants are wary of signing annual medical records releases, researchers say.

Their reluctance is leading to increased concern that without comprehensive laws barring insurance and employment discrimination and protecting the privacy of medical records, patients will shun similar studies of mutation carriers for other diseases. Francis Collins, the director of the National Center for Human Genome Research, says he is "really concerned" about the implications of the recruiting problem with the Pennsylvania study, which hopes to recruit 500 participants over five years.

San Francisco. Representatives from 50 countries attending the first International Summit of National Bioethics Advisory Bodies in San Francisco last week heard pleas for increased sensitivity to non-Western cultures. Solomon Benatar, director of the Bioethics Centre at the University of Cape Town, complained that geneticists had shown a lack of understanding of the history of the African continent, and done little to reassure that the discrimination of the past would not continue in a new form.

Ren-Zong Qui, from the programme in bioethics at the Chinese Academy of Social Sciences, asked delegates to the meeting, which was organized by the US National Bioethics Advisory Commission, to keep an open mind over the law on maternal and infant care in China. He said that the law was intended not as a eugenics policy, but to reduce misery by providing the information, education, counselling and services needed to prevent genetic diseases. □



**Pharmacia
Biotech**
Uppsala, Sweden. (And the rest of the world)

The real threat from antibiotics

SIR — In your leading article “Distrust in genetically altered foods” (*Nature* 383, 559; 1996) you side with the British Advisory Committee on Novel Foods and Processes (ACNFP) on the issue of an ampicillin resistance gene in some strains of transgenic corn. The ACNFP had decided that the ampicillin resistance gene in the transgenic corn posed an “unacceptable risk” because of the possibility that it might be transferred from the corn genome into the genomes of bacteria found in the intestinal tracts of animals and humans.

Following the ACNFP decision, a group of experts was convened in Talloires, France, in September to consider the issue. The meeting was convened by the Foundation for Nutritional Advancement, a private foundation associated with Tufts University in Massachusetts, and was attended by a small group of microbiologists and food safety experts from the United States, the United Kingdom and several other European countries. The unanimous conclusion of this group, of which I was a member, was that the ampicillin resistance gene in the transgenic corn posed no significant health hazard to humans or animals.

This conclusion was based not only on the fact that the probability that the gene would be transferred from corn to bacteria was negligible, but also on the fact that, even if such a transfer occurred, it would have no clinical impact. The ampicillin gene in the transgenic corn strain is the *bla* gene that is present on pUC19 and other plasmids used by molecular biologists. This gene, which encodes a β -lactamase, was originally cloned from a clinical strain isolated in the 1960s. This type of resistance gene poses no clinical problems today because there are many antibiotic formulations that easily control strains producing this type of β -lactamase.

By contrast, the β -lactamase genes that are currently causing problems in hospitals are modern genes that have evolved extensively during the past few decades to the point where they confer resistance not only to a wide variety of β -lactam antibiotics but also to β -lactamase inhibitors that have been used to ‘recycle’ antibiotics like ampicillin. Additionally, a new type of resistance to β -lactam antibiotics that is different from β -lactamases (mutant penicillin-binding proteins) is causing resistance problems in the Gram-positive bacteria.

Finding the old-style *bla* gene in a hospital isolate today would evoke yawns rather than cries of distress. Moreover, such a gene would most likely have been transferred from other hospital bacteria that carry the gene on transmissible genetic elements. The ACNFP failed to consider the clinical impact of a transfer of the *bla* gene from corn to bacteria in its analysis. Instead, it

seemed to have assumed that all antibiotic resistance genes are equally dangerous, which is definitely not the case.

Unfortunately, *Nature* chose to focus attention on the extremely minor threat posed by transgenic corn while ignoring another European regulatory decision that is far more likely to have an impact on human health. In May, only a few months before the ACNFP decision, the European Scientific Committee for Animal Nutrition (SCAN) approved the continued use of the antibiotic avoparcin as a feed additive for farm animals. Avoparcin is an analogue of vancomycin and is known to select for resistance genes that confer resistance to vancomycin. Vancomycin resistance in Gram-positive bacteria is one of the most serious resistance problems currently encountered in large US and European hospitals, where vancomycin is sometimes the only antibiotic left that is effective against multiply resistant strains of *Staphylococcus aureus*.

Certainly, there is room for argument about the extent to which feeding avoparcin to farm animals — and the concomitant exposure of farm workers and their bacteria to antibiotic selection — might contribute to an increased incidence of vancomycin-resistant clinical isolates, but this seems to me to be a far more serious issue than the remote possibility of transfer of an ampicillin resistance gene from corn to bacteria.

In the last paragraph of the leading article, you expressed concern about “deep-rooted cultural fears of genetic manipulation” on the part of the public and stressed the importance of generating consumer trust and confidence in the new genetically engineered foods. I do not know whether, as you contend, the seed companies are behaving in a way that increases consumer distrust of their product. I do know, however, that we as scientists need to do a better job of communicating scientific issues to the public.

In my view your leading article is a case study in how misguided scientific emphasis can help to increase public confusion and anxiety about genetic engineering and its products. By choosing to give precious space to what is at best a very minor safety concern, while ignoring the real antibiotic resistance problems — such as the continued abuse and overuse of antibiotics by physicians, over-the-counter sale of antibiotics, and use of antibiotics in animal feed — *Nature* is sending the wrong message to the public about the forces that are driving the increase in antibiotic resistance.

Abigail Salyers

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Act now on CO₂

SIR — The main theme of the study on atmospheric CO₂ you published earlier this year by Wigley *et al.* was that “[p]athways involving modest reductions below a ‘business-as-usual’ (BAU) scenario in the early years followed by sharper reductions later on were found to be less expensive than those involving substantial reductions in the short term”. The study also argued that “an immediate departure from the BAU path is not necessarily required for concentration targets of 450 p.p.m.v. and above”.

Wigley is surely correct in stating that the Wigley *et al.*¹ study does not itself advocate a delay in cutting carbon dioxide emissions².

Precautionary action starting now is advocated by various organizations, including the World Energy Council. The reasons include lead times, building up technology know-how and capacity building more generally, financing requirements, and the gradual retirement of the existing capital stock. The potential for increasing energy efficiency, accelerating non-fossil fuel availability and cleaner fossil fuel provision and use is immense. Even action now will, in reality, bring only modest reductions below a BAU scenario in the early years. The results will snowball. The problem now is that not even the industrialized countries are, in aggregate, reducing their carbon dioxide emissions — as the chart you recently produced amply demonstrated².

Michael Jefferson

World Energy Council,
34 St. James's Street,
London SW1A 1HD, UK

1. Wigley, T. M. L., Richels, R. & Edmonds, J. A. *Nature* 379, 240–242 (1996).

2. Masood, E. *Nature* 382, 103 (1996).

Hooked on Nature

SIR — Mark Griffiths suggests six components that “need to be fulfilled if a behaviour is to be defined as ‘addictive’” (*Nature* 384, 18; 1996). My scientific activities fulfil all six components, and my weekly reading of *Nature* fulfils three.

Having experienced signs of withdrawal, ensuing interpersonal conflicts and periods of relapse, I have long suspected this addiction. However, I need to be convinced that it “should be treated no differently from the better known chemically based addictions”.

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Letters submitted for Correspondence should be typed, double-spaced, on one side of the paper only, or e-mailed to corres@nature.com

Tradition stands in the way of promotion

Gerhard Neuweiler

Germany's outdated system for promoting postdocs frustrates the career ambitions of many promising researchers. Worse still, it is preventing universities from competing as well as they should in the international science race.

HE is a 35-year old postdoctoral researcher who has worked in highly regarded laboratories. He has a long list of publications, has taught at two universities and won excellent marks in student evaluations. So you might expect him to be one of the most promising applicants for a professorship.

Not so in Germany, because he has failed to gain the academic grade of habilitation. By awarding habilitation to a postdoc, the university faculty testifies that, according to unwritten standards, the applicant is capable of doing good research, and of teaching. The applicant has to submit a major piece of research, or his publications plus a summary of achievements and a projection of future research. He also has to give a lecture to the faculty.

When the faculty grants habilitation, the *Habilitation* becomes a member of the faculty, gaining the right to accept students and to teach. But there is considerable variation in the standards of selection since each faculty sets its own procedures.

Some east European countries are considering introducing a habilitation system to improve the professionalism of their young university scientists. They should be warned that the German system has serious drawbacks. It brings personal dependencies and subordination. And it retards young scientists' careers without improving the quantity or quality of their research. In fact, habilitation might tell you more about the faculty granting it than about the quality of the research and teaching of the scientist being considered for a vacant post.

Habilitation in Germany is, at best, a mixed blessing. Postdocs depend on the goodwill of their supervising professor and faculty as to when they should apply for habilitation. Habilitation does not guarantee tenure but only opens up the chance to be considered by a searching committee. Yet the law does not strictly demand habilitation as a precondition for elevation to professor. It is simply a rubber stamp, testifying that the faculty considers the *Habilitation* fit for a university career.

The practice was introduced early last century with the successful Prussian university reforms by Wilhelm von Humboldt. Before then, everybody who had a PhD had the right to lecture on any subject they chose. This invitation to dilettantism became intolerable. Habilitation gave faculties the right to select those they considered qualified, and to specify the subject of

qualification. Humboldt's reform resulted in a university monopoly for science and science qualifications.

Since then the way research is performed has changed tremendously. With the foundation of the Max Planck Society and other national research institutions, universities lost their science monopoly, but they have retained their monopoly on qualifications (PhDs and habilitation). Much basic research is done outside universities; there are Europe-wide research facilities, teamwork across continents is a daily occurrence, and international compe-



tion requires quick publication of results. A young scientist's international reputation, based on publications and work in internationally known labs, has become a more quantifiable and more significant feature than a habilitation certificate awarded by a faculty less familiar with the current international state of the field of research.

Today habilitation creates more problems than it solves. The average age of *Habilitation*s grew from 37 in 1970 to 40 in 1995. The most important reasons for this deplorable development are that people are gaining their PhDs later (average age 33) and that postdocs are taking an increasing load of services and teaching. Habilitation has become a career trap: faculties prefer good young applicants for professorships, and give their own postdocs a chance to apply only after habilitation, at an age when their most creative period is ending. Paradoxically, habilitation keeps scientists in a long period of dependency while they are asked to prove their ability to do independent research.

Habilitation is no longer a reliable indi-

cator of excellence in research and teaching. Faculties find it difficult not to grant habilitation to somebody who has grown older while rendering important services and teaching to the faculty and thereby neglecting his own research. After reunification many young west German scientists with habilitation have found professorships with tenure in east German universities. Now faculties in the east realize that habilitation is not always a guarantee of high standards. This disillusionment is one of the reasons why Dagmar Schipanski, the head of *Wissenschaftsrat*, Germany's science council, is calling for non-tenured professorships (see *Nature* 384, 204; 1996).

Habilitation is centred on an individual's record of research and is not shaped for modern methods of teamwork. Single-author research papers have become rare, and faculties have difficulty in pinning down an individual's contribution. Habilitation does no justice to the ability of an applicant to work in a multidisciplinary team. It no longer promotes efficient and internationally accepted research methods.

Habilitation is now a questionable procedure for promoting excellence in science. The practice does little to boost the international reputation of German universities. The spectacular success in science of US and UK universities is perhaps the most convincing argument that other practices may be more effective.

There is nothing wrong in critically evaluating a postdoc career. But why not leave to the selection committees the judgement about who is best for the job on offer? In Germany an open discussion on how best to promote young scientists is confounded by two centuries of tradition and anxieties about losing one of the last strongholds of academic autonomy. After more than two years of discussions, *Wissenschaftsrat* could not agree on alternative ways of promoting postdocs for lifetime university careers. It recently recommended adhering to the status quo. After participating in these talks, a government official advised his colleagues: "whoever lays hands on habilitation will fail in university policy". If he is right, this will do nothing to enhance the competitiveness of young German researchers in the global world of science. □

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Silicon integrated circuits shine

David A. B. Miller

Mixing light-emitting diodes and ordinary electronics on the same silicon chip could create new possibilities for cheap, smart optoelectronics, and inspire new applications.

ACCORDING to Gordon Moore of Intel, we now make more transistors per year than raindrops fall in California, and it costs less to make one than to print a character on this page. The reason is the astonishing power of silicon electronic integration technology to manufacture sophisticated yet cheap integrated systems. But silicon has historically had one embarrassing failing—it cannot emit light. Recent work has raised hopes that there may be ways to get useful light from silicon, but until now there has been no cheap way to integrate such silicon-based light emitters with silicon electronics.

The paper on page 338 of this issue¹ by Hirschman *et al.* from the University of Rochester reports the successful integration of silicon-based light emitters into silicon electronics in a way that appears compatible with mass-manufacturing techniques—an important step in turning on the power of silicon integration technology for a broad range of new optical applications. In doing so, the group have addressed the ‘mundane’ issues of device lifetime and integration that can be so crucial in turning a scientific curiosity into a viable technology.

The very visible failing of silicon to emit useful light has been all the more galling because optoelectronics—the combination of optical and electronic functions—is becoming crucial in handling the information that ordinary silicon technology processes. Optoelectronic light emitters or modulators are essential for the visual display of changing information. Laser diodes read compact disks and drive laser printers. Optics already dominates in sending information over long distances. It is used in networks over shorter distances as

demand for information rises, and may be used increasingly for interconnection within computers. And optical isolators are already used inside electronic apparatus to avoid voltage isolation problems.

There is little reason, however, to develop silicon light emitters merely as substitutes for existing individual devices.



An array of silicon displays. The chemical stability of oxidized porous silicon that allows integration with electronic components also allows many optical emitters to be created on a single silicon wafer. Here there are more than 150 seven-segment displays, each 2.5 by 4 mm. Reflection and emission images are superimposed to show electrical probes in contact with one display, and another one lit up.

The ‘III–V’ semiconductors, such as gallium arsenide, are naturally very good light emitters, and are the basis of a mature, inexpensive technology that is a high-volume business, with more than 30 billion devices manufactured each year. III–V light emitters are so efficient that they are starting to replace tungsten light bulbs, for example in car stop lights. But the benefits of silicon-based light emission probably lie not in competing with silicon’s brilliant III–V cousins, but instead in cheap integration with silicon electronics.

The reason why silicon does not emit efficiently is very basic. Silicon is an ‘indirect-gap’ semiconductor. To conserve momentum, the silicon crystal has to vibrate to allow a photon to be emitted, a requirement that a ‘direct-gap’ semiconductor (such as gallium arsenide) does not have. A variety of valiant attempts to solve this problem have failed, though there are some promising approaches using quantum-confined microcrystals². In

1990, however, Canham³ set the field alight by observing that silicon could give out relatively bright, visible, photoluminescent emission when its surface was made porous through a simple hydrofluoric acid etch.

In photoluminescence, the material is excited using short-wavelength light, though the precise mechanism of the porous-silicon photoluminescence is still not entirely clear. Electrically exciting the emission, for example by incorporating the porous silicon in a diode and driving current through it, is also possible, and is generally more useful for devices. In electrically driven porous silicon, the mechanism of the emission may involve very highly excited electrons (or their positive equivalents in semiconductors, holes) forced into the tiny, porous silicon structures, generating photons in various possible ways as they lose energy in a kind of ‘avalanche’ process⁴.

In the past year or so there have been encouraging improvements in the efficiency of such porous-silicon light emission⁵. Early attempts at electrically driven emitters, though encouraging, had several kinds of practical problems, including slow turn-off times (as long as milliseconds), and short useful lifetimes. Part of the lifetime problem may be that the surface of the porous silicon is not very stable chemically, with silicon–hydrogen bonds being easily broken, leading to ‘dangling bond’ surface states. These states are a common problem in many light-emitting devices because they often lead to undesirable, nonradiative recombination of electrons and holes.

Two separate approaches reported this year, one from a Belorussian–Italian group⁴, and the second from Rochester⁶, showed significant improvements in both useful lifetime and speed of response. The first group effectively encapsulated the porous silicon in aluminium and aluminium oxide, whereas the Rochester group oxidized the porous silicon surface. It may be that the emission from these oxidized porous silicon structures is from an entirely new mechanism, involving defects in the silicon oxide.

The latter oxidation approach is used by Hirschman and colleagues¹. Porous sili-

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con becomes more chemically stable when oxidized, and the interface between silicon and silicon oxide is known to have very few of the undesirable surface states that can quench light emission. Silicon oxides are also compatible with the many processes used in silicon-circuit manufacture, and can probably withstand the subsequent processing steps needed for functional circuits, allowing the successful integration demonstrated by Hirschman *et al.*

One caution is that fields like these can become like the search for the Holy Grail. The search inspires, but it may not be clear exactly what to do, or what the actual benefit is, if the Grail is found. For some applications, such as densely packed optical interconnection to and from silicon electronics, even the very best light-emitting diodes are doubtful candidates because of the fundamental inefficiency of incoherent light emission. In this application, integrating III-V optoelectronic devices (such as high-performance modulators or lasers) with silicon circuitry may be more promising, and may incidentally allow interconnections within computers

to keep up with the advance of silicon information processing⁷.

Even after the demonstration from Hirschman *et al.*, there is still some basic technological work to be done — to integrate emitters with the dominant form of silicon electronics (CMOS), for example. But the thrust of the new work towards integration with silicon electronics is well chosen. The benefit of silicon light-emission will probably be in cost-effective, integrated systems, possibly of surprising kinds. Examples could be displays, integrated optoisolators, low-density optical interconnections, or possibly radical applications such as microscopic sensor systems with built-in illuminators.

Certainly, this field is exciting, is progressing fast, and is dispelling doubts that the initially encouraging discoveries might turn to disappointment. There is increasing hope that the light at the end of the silicon tunnel is not an oncoming train. □

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MUSCULAR DYSTROPHY

Utrophin to the rescue

Kevin P. Campbell and Rachelle H. Crosbie

DUCHENNE muscular dystrophy (DMD) is a fatal genetic disease caused by the absence of dystrophin in muscle. The disease is characterized by the progressive loss of muscle strength, and patients usually die by their early twenties of respiratory or cardiac failure. Although great progress has been made in understanding the molecular genetics of DMD, no effective treatment for this devastating disease has yet been developed. Because dystrophin is a structural muscle protein, therapies for DMD will probably involve the replacement of dystrophin or the upregulation of a functionally related protein, such as utrophin. The paper by Kay Davies and colleagues on page 349 of this issue¹ shows for the first time that utrophin can effectively rescue dystrophin-deficient muscle *in vivo*, providing strong support for a therapeutic strategy to fight DMD involving the upregulation of utrophin.

Dystrophin is tightly associated with a large oligomeric complex of membrane glycoproteins that are collectively referred to as the dystrophin–glycoprotein complex (DGC; refs 2, 3). The DGC spans the sarcolemma of skeletal and cardiac muscle, and biochemical and molecular studies have shown that it provides an important structural link between the actin cytoskeleton and the extracellular matrix² (see Fig. 1). The involvement of this cytoskeleton–extracellular matrix connection in

muscle physiology is not fully understood; however, it probably stabilizes the sarcolemma, thereby protecting it from stresses that develop during muscle contraction^{4,5}.

The absence of dystrophin in DMD

patients results in several characteristic pathological features, including muscle-cell necrosis and regeneration⁶, and elevated serum levels of muscle creatine kinase. At advanced stages of the disease, muscle is eventually replaced by fat and connective tissue. On the molecular level, the lack of dystrophin leads to a dramatic reduction in the levels of all of the other DGC components², and loss of the DGC breaks the transmembrane linkage and weakens the muscle-cell membrane. Elegant studies from the laboratory of Jeff Chamberlain^{7,8} have shown that in the *mdx* mouse — a dystrophin-deficient animal model for DMD — restoration of the DGC by transgenic expression of dystrophin is necessary to rescue normal muscle physiology. In particular, the actin-binding and β -dystroglycan-binding domains are required⁸. These findings have been corroborated by the adenoviral expression of dystrophin and internally deleted forms of dystrophin (or minigenes) in *mdx* mice.

Utrophin is structurally similar to dystrophin⁹, and it is mainly expressed at the neuromuscular junction in adult skeletal muscle (Fig. 1), although it is also found at the sarcolemma in fetal and regenerating muscle and, at very low levels, in DMD muscle⁹. Molecular studies of muscle from *mdx* mice have shown that utrophin associates with sarcolemmal glycoproteins to form a complex that is similar to the DGC (ref. 10), suggesting that utrophin can replace dystrophin at the sarcolemma in dystrophin-deficient muscle.

By expressing high levels of utrophin in *mdx* mice, Tinsley *et al.*¹ now demonstrate that utrophin can functionally replace dystrophin (Fig. 2). They show that overexpression of utrophin leads to the restoration of all of the components of the DGC, including the dystroglycan and sarcoglycan sub-complexes. Furthermore, serum creatine-kinase levels and muscle pathology also seem to be corrected, indicating that the restored complex is functional. Overexpression of utrophin even rescues the deterioration of the diaphragm, which is the most severely affected muscle group in the *mdx* mouse¹¹.

The results from the Davies group¹ provide the impetus for an exciting new avenue in DMD-therapy research — the identification of small molecules that increase the expression of utrophin in dystrophin-deficient skeletal muscle. These could be used to upregulate utrophin expression in all

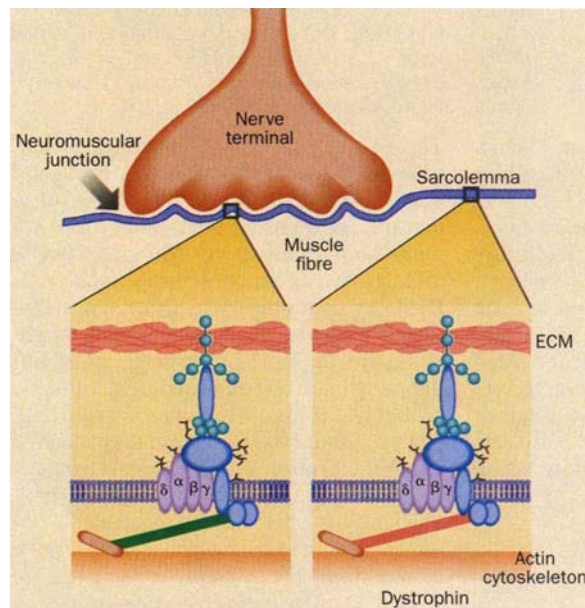


FIG. 1 Localization of dystrophin and utrophin in the muscle cell. The nerve terminal contacts the sarcolemma, creating the neuromuscular junction: expression of utrophin is confined to this region, whereas dystrophin is found throughout the sarcolemma. Both proteins are associated with a glycoprotein complex that binds to the extracellular matrix (ECM).

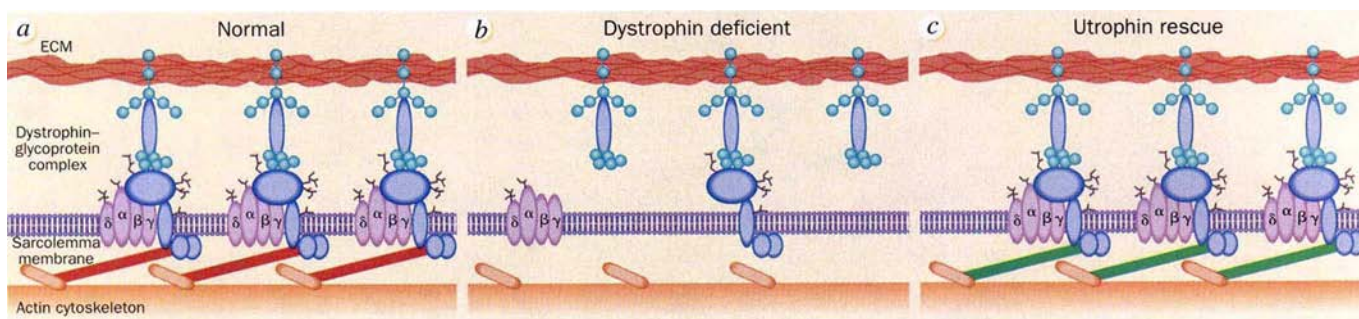


FIG. 2 A possible mechanism of utrophin rescue. *a*, Normal interactions of components of the dystrophin-glycoprotein complex with both the underlying actin cytoskeleton and the extracellular matrix (ECM). *b*, Disruption of the ECM-cytoskeleton linkage that occurs in the absence of dystrophin — as is the case in DMD patients and *mdx* mice. Levels of the dystrophin-associated proteins are also reduced in the absence of dystrophin. *c*, Utrophin, which is structurally similar to dystrophin, replaces dystrophin and restores the glycoprotein complex, maintaining the linkage between the intracellular cytoskeleton and the ECM.

muscles (including those responsible for respiration) and so alleviate completely the consequences of dystrophin deficiency in DMD patients. An advantage of this approach is that it does not require gene replacement, because the patients already have a functional utrophin gene. The next hurdle is to identify a drug that can induce utrophin expression; although this is a rather large obstacle, the upregulation of fetal haemoglobin in sickle-cell anaemia patients sets the precedent for such a therapeutic approach^{12,13}.

Tinsley *et al.*¹ also provide the first evidence to support the use of utrophin and utrophin-minigene transfer as a valid, therapeutic strategy. DMD patients do not express dystrophin, so their immune systems have not seen this protein. This increases the likelihood of an immune response to dystrophin that is expressed from an exogenous gene during dystrophin gene-replacement therapy. The use of utrophin as the transgene will minimize unwanted immunological responses, because utrophin is already expressed in DMD patients. For these types of therapies it will be important to determine the longevity of the utrophin rescue in the *mdx* mice.

There are now several lines to pursue in the development of treatments for DMD — dystrophin gene therapy, utrophin gene therapy and utrophin upregulation. A multifaceted approach to DMD therapy may well be the best course, and a better understanding of the function of the DGC will be invaluable for this. With regard to

the use of utrophin upregulation as a therapeutic strategy for DMD, the next step will be to investigate the elements that control the expression of utrophin in skeletal muscle. For example, what regulates the localization of utrophin to the neuromuscular junction? By altering this regulation, could the expression of utrophin be extended to the sarcolemma? And because utrophin is expressed in the sarcolemma of fetal muscle, could the developmental regulation of utrophin be controlled? Although we don't yet understand the mechanisms controlling utrophin expression, it is possible to screen for small regulatory molecules in skeletal muscle cells. This may be a tremendous task, but it's too exciting a possibility to pass up.

Because the DGC and normal muscle physiology can be restored by the upregu-

lation of a related protein, it may be possible to use a similar treatment for other types of muscular dystrophy (for example, limb-girdle and congenital) that are caused by primary defects in genes for other DGC components (such as the sarcoglycans and laminin-2)². This depends on whether proteins that are functionally related to these components exist. Although the current results do not provide an immediate cure for DMD, they give us one more tool in an ever-growing arsenal with which to attack this devastating disease. □

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INTERSTELLAR CHEMISTRY

Missing link found in space

Stephen Lepp

AFTER more than 15 years of searching, H_3^+ has finally been detected in interstellar space. H_3^+ is the simplest stable polyatomic molecular ion, consisting of three bound hydrogen atoms minus one electron. Its characteristic absorption spectrum was detected by Geballe and Oka (who report on page 334 of this issue¹), along two lines of sight towards young stars embedded in molecular clouds. This detection provides the most direct evidence yet for the ion-molecule reaction networks that are believed to drive the chemistry in interstellar molecular clouds. H_3^+ controls the entire chemistry of these clouds, and the chemistry in turn controls how fast the gas can cool and collapse to form stars². So an understanding of the chemistry is critical to an understanding of star-forming regions.

Interstellar clouds are made mostly of molecular hydrogen (H_2), but traces have been found of nearly a hundred other molecular species³. These include familiar molecules such as water, carbon monox-

ide and ethyl alcohol, as well as many less familiar molecules, including radicals such as OH and CN. Although by number these molecules are a small fraction of the total, they dominate the cooling of the clouds, and are therefore important factors in forming new stars.

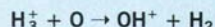
But these clouds are at very low temperatures, around 10 K, at which ordinary chemistry proceeds slowly or not at all. The chemistry must be driven instead by cosmic rays — high-energy particles that stream through the Galaxy and ionize the gas to form H_3^+ . We believe that nearly all of the trace molecules are formed by reactions involving H_3^+ , with the exception of molecular hydrogen, which is formed on grain surfaces.

H_3^+ is made after a hydrogen molecule is ionized by a cosmic ray to form the molecular ion H_2^+ . This then reacts with molecular hydrogen to form H_3^+ and atomic hydrogen. H_3^+ controls the rest of the gas-phase chemistry and produces more com-

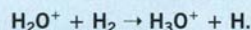
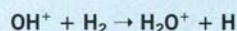
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Making molecules

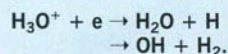
H_3^+ often makes more complicated molecules by beginning with a proton transfer reaction. For example, water, H_2O , and the hydroxyl radical, OH , both result from a string of reactions beginning with:



and continuing to make H_3O^+ via:



The H_3O^+ ion then reacts with an electron to form H_2O or OH :



We believe that most of the complex molecules in interstellar space are made by similar sequences. □

plex molecules (see box) with sequences that begin with the proton-transfer reaction $H_3^+ + X \rightarrow H_2 + XH^+$. Here, X is any chemical species with a greater proton affinity than molecular hydrogen — nearly everything heavier than helium.

That H_3^+ should exist in the interstellar medium was first proposed more than 30 years ago¹. It was first seen in the laboratory⁵ in 1980, and with the spectrum in hand, a number of researchers attempted to find it in interstellar clouds. It was seen in Jupiter⁶, Uranus⁷, Saturn⁸ and possibly in the ejecta of Supernova 1987A (ref. 9), but, being low in abundance and with only a weak emission spectrum, H_3^+ has not been found in interstellar clouds until now.

As an example of how measuring H_3^+ may help us understand the overall chemistry, consider the OH radical. As the box shows, OH is produced by a sequence that starts with atomic oxygen, but it is also removed mostly by reactions with oxygen¹⁰, so its abundance is not sensitive to the atomic oxygen abundance. Instead, it depends on the H_3^+ abundance and the reaction rate coefficients, so simultaneous measurements of H_3^+ and OH could be used to determine the rate coefficients, some of which are highly uncertain. For example, laboratory measurements^{11,12} of the branching ratios in the recombination

of H_3O^+ to H_2O vary from 5 to 33 per cent.

Perhaps the most exciting possibility is the usefulness of H_3^+ as a probe of the ionization rate of interstellar molecules. The formation rate of H_3^+ is directly proportional to the ionization rate times the H_2 abundance, and it is removed primarily by CO — the most abundant of the species that accept a proton from H_3^+ — so measuring the abundances of H_3^+ , H_2 and CO allows a direct calculation of the ionization rate. This is in contrast to the indirect

methods, such as measuring the flux of diffuse γ -rays, which we assume are produced by the same cosmic rays that ionize gas in molecular clouds.

With the detection of H_3^+ , the missing link between ionization and the rest of interstellar chemistry has been found. □

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POLYMERS

When a thick film is thin

Paul Calvert

THE behaviour of polymer films less than a micrometre thick has not long been a concern of polymer science because films and coatings are usually much thicker. But new applications of polymers in electronics have prompted an interest in the properties of these very thin films. They can be strange: a recent paper on a polystyrene¹, published in *Macromolecules*, shows that the diffusion rate in molten films 100 nm thick is half that in bulk polymer, implying that a polymer film effectively becomes 'thin' when it is still many hundreds of atom layers thick, whereas most liquids only show surface effects at thicknesses of one or two atom layers.

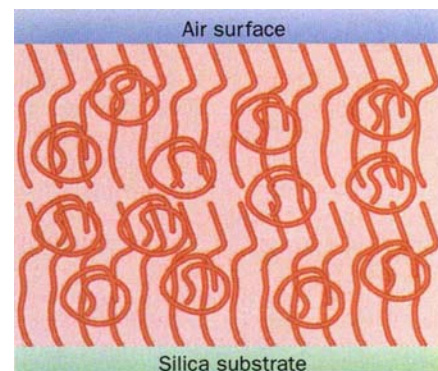
An earlier study explained this as an effect of adsorption². A thin layer of deuterated polystyrene was deposited on a silicon surface and then covered with a thicker layer of normal polystyrene. At 150 °C, the depth profile of deuterium in the film showed that diffusion away from the silicon surface was slower than diffusion away from the air surface. That was attributed to the polymer chains being adsorbed to the silicon so that they only became detached slowly.

In the new experiments¹, Frank *et al.* traced diffusion by attaching a fluorescent dye to polymer chain ends. The film, on a silica substrate, was then bleached in stripes 4 micrometres wide using laser light shone through a mask. As chains diffused into the exposed region from the shadows, the fluorescent signal returned, so diffusion in the plane of the film (rather than away from the surface) could be measured as a function of film thickness. The value was the same as for bulk polymer down to 200 nm, but then diffusion slowed by half as the thickness decreased to 100 nm. This cannot be explained as slow detachment from the surface, so why does it happen?

Long-chain polymers take on a random coil conformation that occupies a sphere. For the chain length in this sample, the radius of gyration is about 3 nm. So if we think of the chains as 3-nm fish in a pond about 100 nm deep, it is hard to see why

their motion should be much affected by the depth. It is possible that the dye units are adsorbing strongly to the substrate, but that would have little evident effect because immobile dye would not be counted.

The critical film thickness, 100–150 nm, is comparable to the fully extended chain length of 120 nm. Without any detailed



Polymer fish in polymer reeds. If a large number of chains are adsorbed at the surfaces, they could hinder the other, coiled chains and explain the slow diffusion observed in some polymer films.

explanation, Frank *et al.* think that somehow the chains are slowed by being unable to straighten fully as they flex between conformations. It could also be that the chains at the silica interface, or at both surfaces, are not coils but are stretched out straight like reeds on the pond bed. Now the remaining coiled 'fish' chains will move slowly through the 'reeds' (see figure). This leaves us with the puzzle of why such a gross distortion of the polymer conformation should occur at these interfaces.

There have already been hints of the long-range influence of polymers at surfaces. Studies in 1984 of polymers adsorbed from solution to mica surfaces showed that attractive forces between the chains went out 50–100 nm, or about three times the radius of gyration of the chain, for both polystyrene in hexane and polyethyleneoxide in water^{3,4}.

But the 'reed' model requires that

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something anchor the reed to the interface. Our knowledge of small molecules tells us that solutes only segregate at a surface when there is a big difference in intermolecular bonding energy between solute and solvent. The classic example is the tendency of detergents to form a monolayer on water in order to get the hydrocarbon tails out of the water.

In polymers, however, the entropy of mixing between components of a blend is small, so quite small differences in structure can drive phase separation. A number of recent papers show that small structural differences can also cause segregation to a surface. Secondary-ion mass spectroscopy has shown that for matched-molecular-weight blends of crystalline and amorphous polymers, the amorphous material occupies most of the surface⁵. There is also evidence of a 10-fold increase in the concentration of chain ends at a free surface⁶. If the polymer surface is dense in chain ends, the chains will have to stretch into the films, like closely planted reeds, and the influence of the surface will be detectable a full chain length away.

The current interest in thin polymer films is driven by new applications of polymers in electronics: the search for higher-resolution photolithography for making integrated circuits; the use of oriented polymer films for controlling alignment of liquid crystals in displays; and the possibility of using high-temperature polymers as insulating layers in integrated circuits and semiconductor packaging. Any such applications require control of adhesion, itself a surface effect.

It may be that these studies will also shed light on some much older problems. It has long been believed that the reason carbon black improves the wear properties of rubber is that the rubber chains adsorb to the carbon-particle surface. This bond is just strong enough to stiffen the rubber but weak enough to slip and absorb energy at high stress. Silicone rubber is relatively feeble because we have never found anything that it sticks to well enough. Conversely, organic/inorganic hybrid plastics have failed so far because they combine the stiffness of plastic with the toughness of glass, rather than the other way round. This may reflect interfacial adhesion that is too strong. The idea of advanced electronics research fostering developments in something as traditional as the tyre business has a certain appeal. □

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Fire damage soils our forests

Peter D. Moore

THE impact of fire on an ecosystem is invariably visually damaging and yet it may be ecologically constructive. As a management tool for manipulating ecosystems, fire has been used at least since Mesolithic times, and it may have been exploited long before that as a hunting technique in driving game. But what fire actually does to the ecosystem — particularly to the soil — is still poorly understood. One of the products of fire, charcoal, which may be produced in large

while, the charcoal particles that are produced from burning biomass and litter may be scattered by the wind or become washed into soils, especially in valley sites.

The physical effects of charcoal in the soil profile have been investigated by Mallik, Gimingham and Rahman⁴, who found changes in the water infiltration, water retention and porosity of soils following fire. In effect, the fine, relatively inert, charcoal particles enhance the water-retentive properties of a soil

and can make a sandy soil behave almost like a clay.

This is of great ecological significance in valley sites, where a change in the physical properties of the soil following a fire can result in poor drainage and the development of wetland communities. Charcoal redistribution in soil profiles may even provide the waterlogged conditions necessary for the initiation of peat formation⁵.

Soil biology is also known to be affected by fire — microbial populations are often stimulated into renewed activity fol-

lowing burning. During a fire there is a steep gradient in temperature within the soil profile, so the impact on microbial activity is usually put down to the flushing of chemicals from the soil or the supply of additional carbon compounds associated with burning. Before a fire, some organic chemicals (for example, tannins) suppress the growth of microbial populations. This could restrict useful microbial functions in the soil, such as nitrification, and it has been proposed as a mechanism by which successional processes (leading to the development of a higher biomass ecosystem) might be held back⁶. Fire may release this restriction by burning off these organic compounds to allow new developments in both soil chemistry and vegetation. Because such chemicals have a strong ecological influence in many ecosystems — both indirectly through the soil microbes, and as a direct result of their action on plants and seedlings⁷ — the role of fire in interrupting this kind of suppression may be widespread.

Zackrisson, Nilsson and Wardle¹ propose a different means by which fire can release ecosystems from the chemical domination imposed by the plants that produce plant- and microbe-suppressing agents. They suggest that charcoal

quantities, is generally regarded as an inert addition to soils that will have physical rather than chemical effects. Yet it is well known that charcoal can adsorb organic molecules such as phenols, and these, in turn, are important compounds in soil ecology. In the latest issue of *Oikos*, Zackrisson, Nilsson and Wardle¹ have shown that because of its distinctive physico-chemical properties, soil charcoal could have profound effects on the development of vegetation and ecosystem succession following fires in high-latitude coniferous forests.

Most studies of the consequences of fire on soils have concentrated on such effects as the release of nutrients from biomass and litter; the loss of organic matter in the surface layers of the soil; the resulting loss in cation-exchange capacity; and the general rise in pH following cation flushing into the soil². The temporary enrichment of soils after a fire is often evidenced by the invasion of relatively nutrient-demanding plants, for example the fireweed *Chamerion angustifolium*. The fire-driven consumption of organic materials from the surface (or duff) of boreal forest soils also leads to enhanced germination of seeds and the establishment of seedlings³, which have easier access to the mineral soil in the absence of surface litter. Mean-

IMAGE
UNAVAILABLE
FOR COPYRIGHT
REASONS

Yuri Shibnev/Planet Earth Pictures

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POLLUTION

Recipes for cleaner air

William J. Cooper and L. Anita Holloway

adsorbs phenolic inhibitory compounds (including the tannins), and in this way both plant and microbial activities are stimulated. They have studied a forest site in Sweden, where a canopy of Scots pine (*Pinus sylvestris*) has a dwarf-shrub understorey in which crowberry (*Empetrum nigrum* ssp. *hermaphroditum*) is abundant. Crowberry is a distinctly flammable plant because it produces phenolics, which inhibit the growth of tree seedlings and affect the function of the mycorrhizae.

Having collected soil samples from a range of sites and hand-sorted charcoal particles from the soil profiles, the authors made water extracts of dried crowberry leaves and shook some of these extracts with charcoal. They then tested the residual solution by means of a bioassay using aspen seeds, and found that the charcoal from recently burned sites had a high capacity for reducing the ability of the crowberry extract to inhibit germination. Charcoal from older sites was considerably less effective. For example, charcoal from a site that had remained unburned for the last 100 years was about eight times less effective than freshly generated charcoal.

Zackrisson and colleagues also used charcoal that had been specially prepared by burning crowberry stems, and they showed that by sequential additions of this phytotoxic extract they could eventually saturate the charcoal with phenolics. This could account for the inefficacy of soil charcoal from sites that had been burned some time ago. However, incubation of saturated charcoal with fresh, biologically active humus reactivated the charcoal as a result of microbial degradation of the phenolics.

Observations on the tree basal areas — a crude index of tree biomass — in Swedish boreal forests indicate that during the first hundred years of succession following a fire there is a rapid rise in the basal area, after which there is a general decline that may last for a further 200 years in the absence of fire. So fire evidently has ecologically rejuvenating qualities. Many factors are involved, and the phenolic-cleansing effect of soil charcoal is one more to add to the list. □

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EVEN in countries where levels of air pollution are falling, contamination of the atmosphere remains a serious environmental problem. Take the United States. The most recent *Toxic Release Inventory* data¹ issued by the Environmental Protection Agency show that, in 1994, air emissions of chemical pollutants in the United States totalled 706 million kg — putting the figure as 706,000 metric tonnes makes plain the size of the burden imposed upon the atmosphere. The rise over 1993 (see

Year	Total releases (million kilograms)
1988	1,040
1989	975
1990	875
1991	771
1992	717
1993	635
1994	706

table) is probably due to changes in reporting requirements, and in general there has been a steady decrease in air emissions in recent years. Nonetheless, there is plenty of scope for improvement through application of new control technologies. It is impossible to extrapolate to the world as a whole from data obtained in the United States alone; however, the problem worldwide is probably at least twice that of the US emissions.

In this context, the uranium-oxide-based catalysts described by Hutchings and co-workers on page 341 of this issue² look promising. They offer a potential treatment technology that can destroy volatile organic compounds in the gas phase at much lower temperatures and costs, and with greater destruction efficiencies, than can other catalysts or high-temperature thermal oxidation. Their study looked at several different classes of organic chemicals that are potential atmospheric pollutants, especially chlorine-containing molecules such as chlorobenzene and chlorobutane.

Emissions of air pollutants are split into those emanating from non-point sources (emissions lost during a manufacturing process other than through a smoke stack) and those from point sources ('controlled' emissions, emanating from the stack as a point source of pollution). The 1994 *Toxic Release Inventory* indicated that, of the 520 million kg of industrial stack emissions in the United States, about 70 per cent was composed of organic compounds that could be destroyed using catalytic processes. The agents described by Hutchings *et al.* would certainly be applicable in the

clean-up of stack emissions, and the comparatively low temperature (350 °C) necessary for destruction would lead to lower energy costs — a prerequisite for the willing adoption of any new pollution-control technology by industry. Moreover, at temperatures such as this, and on the assumption that no harmful reaction by-products are formed, this would not be classified as an incineration process. In consequence, fewer regulatory hurdles would have to be overcome before a plant employing these catalysts could be approved for operation.

The catalysts may also have applications in areas other than stack emissions. For example, cleaning up contaminated land often involves the use of soil vapour extraction — which acts rather like a large vacuum cleaner — to remove the more volatile compounds from soils or other contaminated media. However, to effect a complete treatment, a second process is required to destroy the compounds that result from vapour extraction. Thermal oxidation has generally been employed in the past, but the uranium-based catalysts might be much cheaper. As the authors point out, "the challenge is to provide effective technology at a cost that will not stifle economic growth".

Full commercialization of a new idea or technology is rarely straightforward, however. The process usually takes longer than expected, for each situation presents unique problems — it is no longer the case that if you build a better mousetrap, the world will beat a path to your door. One possible stumbling block for these new catalysts is that they are uranium-based. Even though they are not radioactive, and similar catalysts have been employed for other purposes for some time, the public will have to be convinced that these compounds are safe. In fact, the most likely place for the initial acceptance of catalysts such as these will be in industry, where, when safety is assured, cost considerations are paramount. This contrasts with situations where public acceptance of a new process, or lack of it, is the main determinant, as for instance in the US Superfund process for the clean-up of hazardous waste. Adverse public reaction to several technological solutions for cleaning up hazardous waste-sites prompted the US Environmental Protection Agency to make it mandatory for public hearings to be part of the process of seeking cost-effective solutions for the Superfund.

Another possible pitfall on the road to commercialization is the creation of toxic by-products during the catalytic process. Hutchings *et al.* have addressed this issue, and find that no reaction by-products are formed for any of the pollutants that they

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The changing sizes of genes

Brian Charlesworth

David Hoffman/Environmental Images

IMAGE
UNAVAILABLE
FOR COPYRIGHT
REASONS

Air of concern.

studied. The authors carried out experiments with several of the organic compounds under consideration; they show that all of the carbon is converted to oxides of carbon, and that any chlorine present formed hydrochloric acid (for which many commercial-scale control technologies are available). Further studies will have to address similar questions when mixtures of more than one compound are present in the gas stream. Hutchings and colleagues' experiments were conducted under controlled conditions, and on one compound at a time. The ability of the family of uranium-based catalysts to treat different types of organic compounds, individually and in mixtures, will be important in determining the market niche that it fills.

Finally, a feature that Hutchings *et al.* briefly describe, but do not expand upon, is catalyst deactivation. This is important in developing a cost-effective treatment process, as well as in implementing it in the clean-up of hazardous sites — in particular where the air stream to be treated is likely to contain several different organic compounds, with concentrations varying with time. Again, additional studies will be necessary before the technique can be applied on an industrial scale.

So plenty of work remains to be done. Nonetheless, the potential is clear — these catalysts could play a pivotal role in realizing the overall goal of cheaper, more reliable pollution-control technology for gas-phase emissions of organic compounds. □

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DIFFERENCES in genome size among species bear little relationship to differences in numbers of coding sequences (the 'C-value paradox')^{1,2}. Because most of these differences are caused by variation in the numbers of copies of repetitive DNA sequences, discussions of the evolution of genome size have centred on the mechanisms that regulate the abundances of such sequences^{2,3}. But the lengths of genetic elements, rather than their numbers, may also change in evolution and alter the total amount of DNA in the genome⁴⁻⁷.

On page 346 of this issue⁸, Petrov *et al.* provide further evidence that this second process contributes to genome evolution. But they also make the provocative claim that mutational biases in the direction of length changes may cause differences between species.

Their work starts with the observation that the class of transposable elements which lack long terminal repeats, but transpose by means of reverse transcription of RNA, are vulnerable to truncation of their 5' ends during transposition, and often cannot transpose⁹. Petrov *et al.* argue that the sequences of such defective elements will be subject to strictly neutral evolution, due to lack of selection on their ability to transpose.

The authors have studied the sequence evolution of part of the reverse transcriptase gene of a *Drosophila* element of this type, *Helena*. By constructing a phylogenetic tree for 18 different copies of *Helena* from eight different species of the *D. virilis* group, they classified the nucleotide substitutions that have occurred into changes that are placed on the internal branches of the tree, and changes assigned to the terminal branches. (The terminal branches are defined simply as the sections of the tree that lie between each tip and the first nodes connecting them to the rest of the tree; the internal branches form the rest of the tree; see Fig. 3 of ref. 8, page 348.) Petrov *et al.* argue that many of the terminal-branch substitutions are likely to have occurred after transposition of the copy in question, in defective copies which are no longer mobile.

This was tested by comparing the relative rates of nucleotide substitutions that are either silent, or result in amino-acid replacements, for the two classes of change. These events are equally frequent among the terminal-branch changes, as would be expected if selection is no longer acting on the reverse transcriptase (which is required for transposition). Similar results have been obtained for an element with long terminal repeats, isolated from a group of parasitoid wasps that includes *Nasonia vitripennis*¹⁰. Petrov *et al.* then

examined the evolution of length changes in the reverse transcriptase gene. By reference to the consensus sequence, these appear to be due entirely to deletions, of up to a hundred bases or so. Each deletion is unique, consistent with its having arisen in a defective element subsequent to transposition. Deletions have accumulated on the terminal branches of the phylogenetic tree, at a rate that is about 16 per cent of that for nucleotide substitutions.

These data provide important new quantitative information on the nature of DNA sequence evolution when unconstrained by selection. But Petrov *et al.* make a further claim, that *Drosophila* DNA is inherently prone to deletions at a relatively high rate. They suggest that this may explain such features of the *Drosophila* genome as its dearth of pseudogenes: if deletions accumulate at a high rate in non-functional sequences, then these will gradually disappear over evolutionary time. Extrapolating further, they propose that "differences in genome size may be determined primarily by the variation in the genome-wide deletion rate".

This claim is debatable, however. First, in most taxa, variation in numbers of highly repeated sequences must far outweigh any contributions from changes in lengths of non-coding sequences such as introns and pseudogenes, simply because of their numerical predominance¹⁻³. Second, there is other evidence from *Drosophila* to suggest that the evolution of length changes is a more complex process than one-way mutation to deletions. For example, comparisons of intron lengths between closely related species of *Drosophila* reveal no particular directionality: in some genes, longer introns have evolved, in others, shorter ones^{4,6}. Curiously, *D. melanogaster* appears to be evolving longer proteins than its close relatives⁶. A lack of directionality in length changes is seen for within-population variation of the size of the enhancer region of the *even-skipped* gene in *D. melanogaster*

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and *D. simulans*⁷. This contrasts with a predominance of deletions among changes that have been fixed within species in the *D. melanogaster* species group⁷. Such discordance between within- and between-species variation is strong evidence for positive selection in favour of shorter sequences in this region.

So there is an alternative interpretation of the data of Petrov *et al.* — that there has been selection for deletions, possibly because of an advantage to smaller genome size. The authors consider this possibility, but dismiss it on the grounds that there is no correlation between the total amount of deleted DNA and the number of terminal substitutions; it is not clear, however, why such a correlation is required by the selection hypothesis. A difficulty with the selection hypothesis is that it is hard to imagine that a significant advantage or disadvantage would accrue to a length change that represents one in a million or less of the total number of bases in the genome. But recent work shows that selection coefficients of the order of one in

a million seem to be effective in maintaining codon usage bias in *Drosophila*¹¹. In addition, we know that flies have huge effective population sizes¹¹, so that very weak selection can significantly affect the fate of new mutations¹². This means that selection on small length changes cannot be ruled out *a priori*.

It should be clear from this discussion that we are only beginning to acquire even a crude picture of how the lengths of genes and regulatory regions change during evolution. An understanding of the forces involved in causing these changes must await the collection of more extensive data, and the application of statistical methods that may enable us to disentangle the actions of different evolutionary factors. Results such as those of Petrov *et al.*⁸ should provide a stimulus to this enterprise. □

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ENZYME MECHANISM

DNA helicases get physical

Stephen C. West

THE ability to unwind duplex DNA is a property shared by a diverse group of enzymes, known as DNA helicases, that are involved the replication, recombination, repair and transcription of DNA. These are key biological processes — hence the interest that will attend the publication, by Subramanya and colleagues, of the first X-ray crystal structure of a DNA helicase (page 379 of this issue¹).

DNA helicases use energy from the hydrolysis of nucleoside triphosphates to catalyse the breakage of hydrogen bonds that hold DNA strands together². Some also use this energy to fuel their translocation along DNA. Even though a large number of helicases have been purified and characterized (12 from *Escherichia coli* alone), they have remained somewhat

enigmatic because their cellular functions are varied and the precise mechanisms of action are poorly understood.

Many DNA helicases share structural similarities, the signature being seven distinct and highly conserved peptide motifs. Two of these correspond to the so-called Walker A and B sites involved in binding nucleoside triphosphate. The presence of the seven conserved motifs has led to some proteins being defined as DNA helicases on the basis of sequence alone. Occasionally this has led to a 'wrongful arrest', but more often than not the predictions have proven to be correct. There is also a connection with human disease, in that sequence analysis has shown that the molecular defects responsible for Bloom's syndrome (characterized by

genetic instability and a predisposition to cancer) and Werner's syndrome (premature ageing) reside in genes that may encode DNA helicases^{3–5}.

Subramanya and colleagues describe the monomeric structure of the PcrA helicase, isolated from *Bacillus stearothermophilus*, in the presence and absence of ADP. Because the protein is relatively unknown, this report is unusual: here we have a case where solution of a detailed protein structure has preceded biochemical analysis. But the clues to helicase action are likely to reside in the seven conserved structural motifs, and it is therefore of great interest to see where the conserved motifs lie in relation to overall protein structure.

The crystal structure shows that the protein is composed of two domains (1 and 2), each of which contains two subdomains (A and B). The ADP cofactor is located in a cleft between the two domains and, remarkably, all of the conserved motifs are located around this nucleotide-binding site. The ATP-binding site is therefore ideally situated to effect conformational changes between the two protein domains, thereby coupling hydrolysis of nucleoside triphosphate with DNA unwinding. Such conformational changes are likely to underlie its enzymatic mechanism.

A second unexpected and striking observation of Subramanya *et al.* is that the structures of the A subdomains of the PcrA helicase are similar to that of the ATPase domain of the *E. coli* RecA protein. Indeed, detailed comparison of the PcrA–ADP complex with the RecA–ADP complex⁶ reveals that all of the main residues in the nucleotide-binding site of RecA are spatially conserved in PcrA.

The RecA protein is central to genetic recombination and DNA repair, in that it is directly responsible for promoting ATP-dependent homologous pairing and strand-exchange reactions between two DNA molecules. It also provides the structural unit within which recombination reactions take place, forming a protein filament that acts like a sheath around the DNA (Fig. 1). In this nucleoprotein filament, RecA changes the helicity of the DNA from the

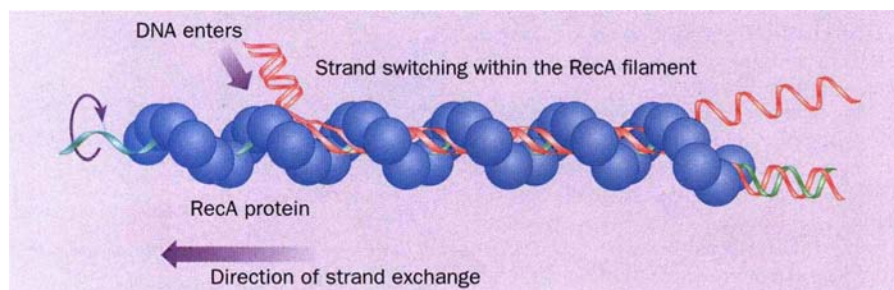


FIG. 1 Model for homologous pairing and strand exchange by the *E. coli* RecA filament. The DNA is extended and lies along the longitudinal axis of the filament. There are six RecA monomers per helical repeat (18.6 base pairs). Pairing occurs within this nucleoprotein structure as the two DNA molecules intertwine. The next step, that of strand switching, involves the breakage and re-formation of hydrogen bonds. The reaction occurs as ATP is hydrolysed and involves conformational changes to each RecA subunit.

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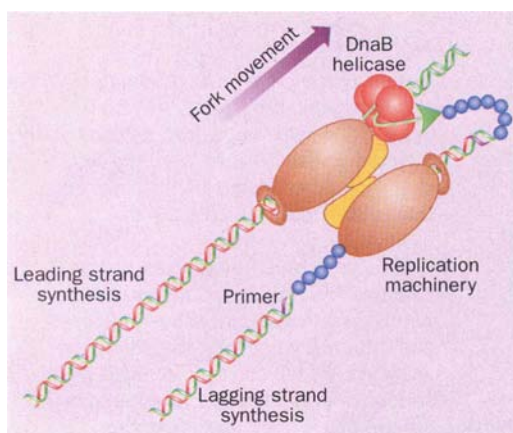


FIG. 2 Model for the action of a DNA helicase during the replication of DNA in *E. coli*. DnaB catalyses DNA unwinding as the replication machinery moves along duplex DNA, facilitating synthesis of the leading and lagging strands of DNA. Strand separation by DnaB is coupled to ATP hydrolysis, as is the enzyme's ability to translocate along DNA and lead fork movement. It is not known whether one or both strands of DNA pass through the central hole in the hexamer ring.

Watson-Crick B-form (10 base pairs per turn of the helix; 34 Å pitch) into an extended structure (18.6 base pairs per turn; 95 Å pitch)⁷. The filament is essential for genetic recombination, and appears to have been conserved during evolution because very similar structures are formed by the yeast⁸ and human⁹ homologues of RecA.

Is the structural relationship between RecA and a DNA helicase significant? Certainly there is little direct evidence to

suggest that RecA acts simply as a helicase during strand exchange, but the actions necessary for strand exchange may not be so far removed from helicase activity as to preclude a common ancestral origin. During recombination, RecA establishes homologous contacts between two DNA molecules, promotes the breakage of hydrogen bonds, and facilitates an exchange of strands between the two paired DNAs. The mechanisms by which these events are accomplished remain controversial (is there or isn't there a triple-helical DNA intermediate?), but they most certainly involve the close intertwining of two DNAs within the filament. Strand exchange is likely to result from a cycle of conformational changes that occur in response to ATP binding and hydrolysis. So the mechanism underlying this process may be related to that by which DNA helicases break hydrogen bonds.

An intriguing aspect of DNA helicase action is that the proteins generally function as multimers, usually dimers or hexamers. Helicases such as *E. coli* DnaB, RuvB and Rho, bacteriophage T4 gp41 and T7 gp4, and SV40 large T antigen all form hexameric ring structures¹⁰ with a central hole through which the DNA passes (Fig. 2). This arrangement is not so different from the structure made by

RecA, as six RecA monomers form one helical repeat and the DNA lies within the deep groove of the nucleoprotein filament (Fig. 1). The ATP-binding sites of Rho, T7 gp4 and RecA are structurally related to that of the α - and β -subunits of the F_1 -ATPase^{11,12}, an apparently unrelated enzyme that converts ADP and inorganic phosphate into ATP during oxidative phosphorylation. The α - and β -subunits of this protein also take the form of a ring-shaped hexamer ($\alpha_3\beta_3$) with six nucleotide-binding sites¹². Moreover, the γ -subunit of the ATPase is positioned within the central cavity of the ring, analogous to the way in which the hexameric helicases bind DNA. The relationship is extended even further by observations indicating that the loops of RecA that interact with DNA correspond to the loops of the F_1 -ATPase that contact the γ -subunit.

Better understanding of the relationships between PcrA and RecA, together with comparisons with other DNA helicases and the F_1 -ATPase, will help to define the mechanism by which ATP hydrolysis and conformational changes are coupled to allow these proteins to carry out their varied enzymatic activities. Who would have thought that the structure of an unknown DNA helicase would have such far-reaching implications? □

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EVOLUTIONARY GENETICS

Genomic revolutionaries rise up

Laurence D. Hurst and Gregory D. D. Hurst

SELFISH elements are heritable factors that manipulate their host's genetics to their own ends and hence spread in populations despite being deleterious. Well-described examples include meiotic drive genes, such as *Segregation Distorter* (*SD*) of *Drosophila melanogaster*. This acts in males to cause wild-type sperm to die, so allowing all the eggs to be fertilized by the sperm bearing *SD* (see figure overleaf). It is usually assumed that selfish elements afflict only a few species, but a series of reports¹⁻³ shows that these factors are much more common than had been suspected.

The frequency of species with selfish elements is central to debates on their potential evolutionary influence. Because such elements are deleterious, their spread can both potentially lead to population extinction or create the conditions for invasion of modifying genes. Consequently, selfish elements are good candidates for the initiators of major transitions in evolution, from the evolution of sex and sexes, to the advent of

meiosis and multicellularity⁴. If such elements are rarities, however, it is hard to argue that they could be of general importance to the maintenance of genetic recombination or to common processes such as speciation.

John Jaenike's report¹ of the frequency of X-linked meiotic drive in fruit flies casts doubt on the assumption that such elements are mere curios. In the course of other studies, Jaenike examined nine species of *Drosophila* in four different species groups. Although he was not looking for unusual sex ratios, he found that in five out of the nine species something was causing the production of all-female broods in some crosses. By classical segregation analysis, he could show that the species have X-linked drive.

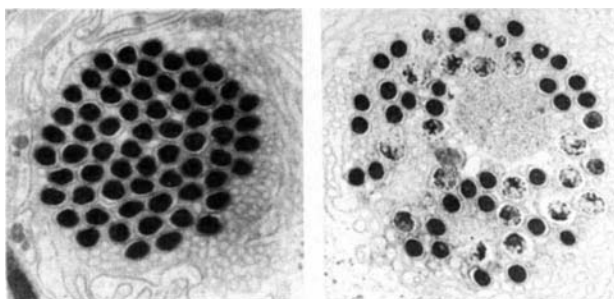
Although this figure is incredibly high, it is a minimum estimate. X-linked drive can potentially cause a population to become extinct, in which case we cannot detect it. Furthermore, Jaenike's method could only find X-linked drive if it is poly-

morphic within the species, but other studies by Hervé Merçot and colleagues² show that the absence of a polymorphism for drive need not indicate the absence of a driving chromosome.

This group examined *D. simulans* in New Caledonia and the Seychelles. Crossing individuals from the islands they found normal sex ratios. But hybrid males (male progeny of island females mated with males of standard strain) often produced heavily female-biased broods when crossed, and Merçot and colleagues showed that this was due to an X-linked meiotic drive gene. Within the island populations, they surmise, the rest of the genome had evolved to suppress the drive, but the suppressors were specific to the region and hence drive emerged in the male hybrids. This not only indicates that drive may well be cryptic but, as the authors point out, it also supports theories of speciation as being driven by co-evolution of drivers and the rest of the genome. Furthermore, evidence from *D. melanogaster* is consistent with the existence of X-linked drive suppressed by genes on the Y chromosome⁵, a story repeated in plants⁶.

Not all selfish elements are so integral to the genome. Some arthropods, for

Signs of selfishness — the effect of the meiotic drive gene *Segregation Distorter* (SD) in the fruitfly *Drosophila*. Both of these electron micrographs show a section through the head region of a sperm bundle ($\times \sim 7,500$). Normally (left) the nuclei in a bundle of 64 developing sperm cells form 64 sperm heads. In flies carrying SD (right) only 32 heads mature, those that will pass on the SD gene. (Reproduced from *Scientific American* February 1979. Micrographs by R. W. Hardy and K. T. Tokuyasu.)



instance, are hosts to bacteria that are exclusively maternally inherited. Because of this transmission pattern, these factors have no evolutionary interest in being in sons and many manipulate their host in one way or another to prevent this from occurring. In certain wasps⁷, for example, there are bacteria that force their female host to reproduce asexually and have exclusively female offspring. This phenomenon has evolved at least five times⁸. In Crustacea some bacterial symbionts have evolved to turn sons into daughters⁹, while in numerous insects there are symbionts that kill sons¹⁰.

The induction of parthenogenesis in wasps and feminization in crustaceans is performed by the same 'species' of intracellular symbionts (*Wolbachia pipientis*). In other species this bacterium induces 'cytoplasmic incompatibility'. Here the bacteria stay in the male but somehow poison the sperm so that progeny sired with uninfected females die (infected eggs develop normally).

Jack Werren and colleagues³ have surveyed 154 species of neotropical insects for *Wolbachia*. Rather than looking for the phenotypic footprint of the selfish element, they used the polymerase chain reaction and dot blot to detect the version of the *ftsZ* gene specific to *Wolbachia*. They found it in 16.9 per cent of species, and in a similar percentage of insects in the United States, and by extrapolation calculate that, overall, between 1.69 and 5.07 million species of insect are infected. Later studies have reported *Wolbachia* in both mites¹¹ and a filarial worm¹². It is not known what proportion of *Wolbachia* infections exhibit or exhibited selfish effects. But given that cytoplasmic incompatibility can potentially induce reproductive isolation between populations, it is reasonable to speculate³ that the spread of *Wolbachia* could be a major cause of speciation.

It is tempting to assert from such findings that selfish elements are common, but often unnoticed. Such a conclusion, however, is wrong, for not all well studied species have selfish elements that have major effects. There are probably good reasons for this, one being that the spread of selfish elements often requires the species to be outbreeding. It is probably

for this reason that yeast, *Caenorhabditis* and *Arabidopsis* harbour only relatively small-effect selfish factors (transposons, for example). The other common laboratory species, drosophilids and mice, are all naturally outbred and commonly have major-effect selfish factors⁴.

The outbreeding rate is not, however, the sole determinant of the distribution of selfish elements. Male-killing elements die when the male dies. For this to be a successful strategy some advantage must be given to the dead males' sisters. This requirement limits the distribution of such factors but also explains why they are prevalent in species with intra-brood cannibalism and gregarious clutches¹⁰. In ladybirds, in which conditions seem near perfect for male-killers, work in our department showed that three out of seven randomly chosen species had the factors. In one species male-killing has evolved at least twice.

There remains one curious species that is both well studied and outbred, but shows very little evidence of harbouring major-effect selfish elements. Although there are descriptions of curious sex ratios¹³ and, occasionally, of weak segregation distortion (for example see ref. 14), there are no convincing reports of such a selfish element in humans. □

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DAEDALUS

Second sight

ALDOUS Huxley, the great prophet of hallucinogenic drugs, had very bad eyesight. He was entranced by chemically induced visions — they were the first sharply focused things he had ever seen. Daedalus is now building on his experience.

The eye is much more than a mere camera. The retina, and its optical pathways to the brain, actively process and interpret what they see. There are specific cells which react to edges of various orientations, to movements in different directions, to differences between signals from the two eyes, to specific spatial frequencies. Farther back in the system, individual objects are recognized and identified. The hallucinogens, says Daedalus, must sabotage this delicate machinery by binding selectively to specific cells. In low doses they can merely enhance colours or shapes. Other drugs seem to target different cells. Some carbamates blur the vision; strychnine in tiny doses sharpens perception; a rare side-effect of ranitidine is to abolish colour vision.

DREADCO biochemists are expanding these observations. They are seeking drugs which, so to speak, will conduct by software the operations now performed in hardware by ophthalmic lenses. A drug which damped down the cells handling low spatial frequencies, or excited those handling high spatial frequencies, would sharpen the vision. One which acted preferentially on the cells detecting specific angles could equalize the biased angular contrast of astigmatism. One which targeted the cells of binocular fusion could merge the images from divergent eyes. Daedalus hopes to perfect a range of 'pharmacological spectacles': drugs to be made up by the optician according to the defects revealed by his eye-test. A simple course of tablets will then give perfect sight, ending the long tyranny of frames and contact lenses.

But Daedalus goes further. He is seeking compounds to affect the deeper visual interpretations. A stimulant for the motion-sensing cells would do wonders for the skills of driving, machining and similar dynamic tasks. Conversely, one which extended the persistence of vision would allow the user to appreciate jerky old films, or see a sequence of slides as a moving picture. A drug which emphasized the differences between straight, curved and closed letters would please proof-readers and dyslexics. A pill which clarified the key differences between human faces would lay to rest many social anxieties, while pills which heightened perception of mislaid keys, or parking spaces, or policemen, would all find market niches.

David Jones

Migration corridor for sea turtles

SIR — Sea turtles and other rare organisms are frequently considered indicators of trends in biodiversity and resource availability. The endangered leatherback turtle, *Dermochelys coriacea*, is the largest turtle and, although it occurs worldwide ranging as far north as the Arctic Circle^{1,2}, little is known of its behaviour at sea. Individuals may be capable of trans-oceanic journeys³ and many are seasonal migrants⁴. Critical details of timing and spatial patterns of oceanic movements, however, have remained elusive until now. Using satellite telemetry techniques, we have tracked leatherback turtles over great distances and have discovered, to our knowledge for the first time, the existence of a distinct turtle migration route across open water. Such an extensive migratory corridor may reflect distribution patterns of marine resources.

Satellite telemetry⁵⁻⁷ has made it possible to monitor the oceanic travels of sea turtles. As the turtle rises to the surface to breathe, the attached transmitter sends a signal to a satellite, which calculates and relays the location of the animal. There has been limited success in attaching transmitters to leatherbacks^{5,7-9} because of such impediments as a pliable oily carapace and extreme conditions encountered during dives which may exceed 1,000 m in depth¹⁰. We overcame some of these difficulties by trailing buoyant, hydrodynamically shaped and pressure-resistant transmitters behind the turtles on a short tether.

Satellite transmitters were attached to eight adult female leatherback turtles after they had laid their eggs on a nesting beach near Playa Grande, Costa Rica. Despite being programmed to last 1 year, transmitter durations ranged only from 3

to 87 days (see table). Seven of these are the longest reported durations for tracking leatherback turtles. Additionally, with travel distances of up to 2,700 km, six of these are the longest detailed tracks of leatherback turtles ever reported.

The most striking feature of the post-nesting migratory behaviour was the related movement among individuals (see figure). Turtles within the same season travelled along similar, and in some cases virtually identical, pathways. In 1992, two turtles were located within 20 km of each other after 37 days and more than 700 km from the nesting beach, even though they left the beach 13 days apart. Similarities in migratory patterns also persisted from year to year. Over the four consecutive years of our study, the tracks of all eight turtles were within a relatively narrow corridor extending southwestwards into the open Pacific. Seven turtles were tracked along this corridor over periods ranging from 29 to 87 days, while traversing net distances from 417 km to 2,780 km. Four of these leatherbacks passed by the Galápagos Islands; one slowed down among the islands for nearly two weeks before continuing into deeper ocean waters.

The probability of all the turtles migrating by chance within such a narrow corridor is low. A turtle leaving the Pacific coast can depart along an arc of roughly 160°. As an example, the migratory movements of olive ridley turtles, *Lepidochelys olivacea*, from a nearby beach were diverse and widespread from year to year, even for nesting cohorts⁷. Despite the vastness of the Pacific, however, the tracks of all eight leatherbacks, when superimposed, were within a southwestward corridor conservatively estimated to be less

Tracking summary of eight leatherback turtles

ID number	Release date	CCL (cm)	Tracking duration (days)	Net distance (km)
2105	15/1/92	164	63	1,639
2106	15/1/92	144	39	820
3560	11/1/93	148	29	417
3561	12/1/93	152	3	67
1109B	11/1/94	150	87	2,780
1110B	13/1/94	153	54*	888
1111C	18/1/95	153	46†	1,451
1112B	19/1/95	151	75	1,886

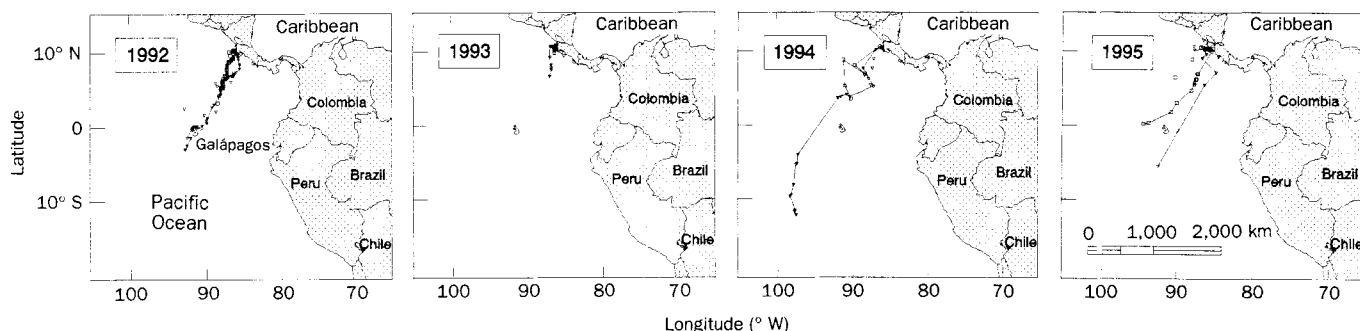
Two nesting cohorts in each of four consecutive years were selected from a nesting beach at Playa Grande, Costa Rica, not more than 5 km apart. After laying their eggs, adult females were restrained on the beach for measurements, tagging and transmitter attachment. Handling time decreased from 5 h to 19 min as our technique evolved. Transmitter packages also decreased from 2 kg to less than 900 g by 1994, when depth sensors were also added. All movements were monitored via satellite for as long as transmissions continued. Distances were calculated using a spherical Earth model. Carapace lengths (CCL) were measured over the curve.

*54 days of data; last position on day 42.

†46 days of data; last position on day 38.

than 500 km wide. This may extend into open waters for as much as 2,700 km, the longest track that we measured.

We do not know how this corridor is maintained through time or how it is detected by the leatherbacks. Because all turtles were still moving at last contact, this open ocean corridor may be only part of the way towards their destination. Migration may be influenced by environmental factors such as currents, bathymetric features⁶, magnetic cues¹¹ or a persistent open-ocean front¹² where turtles converge on a food source. Although we recorded a dive of 744 m, turtles remained predominantly in the upper 300 m of the water column. Thus, it is possible that there is a near-surface feature



Migratory movements of eight leatherback turtles monitored by satellite transmitter after nesting near Playa Grande, Costa Rica (10° 19.0' N, 85° 50.3' W). Pairs of cohorts were tracked beginning in January in each of four consecutive years. Turtle movements and sensor data were monitored daily by TIROS-N series satellite using the ARGOS telemetry system. With this system, transmitter locations are calculated by Doppler shift, and sorted into different categories of certainty. For our turtles, this process assigned 77% of the fixes to a class of unknown certainty. Because many of these unclassified signals are accurate locations, we developed algorithms with which we established our own set of acceptance criteria for the satellite

signals. First, using only the location data of high-certainty categories, an upper limit of travel for migrating leatherbacks was conservatively calculated to be 104 km per day. Using this threshold value as the highest allowable rate of travel between consecutive turtle locations, our algorithms increased the number of plausible locations by more than 61%. Solid symbols, high-certainty locations assigned by ARGOS; lines connect the points included in our own acceptance criteria; open symbols, unknown certainty. The Cocos Ridge runs beneath the first 1,500 km of the migration corridor, extending out to the Galápagos Islands. Four turtles were tracked as they passed the Galápagos and continued along beyond the ridge, into deeper Pacific waters.

that could be detected by shipboard sampling or remote-sensing techniques.

The existence of ocean corridors for sea turtles is important to the development of effective international conservation strategies. Global conservation efforts for sea turtles have focused primarily upon nesting beaches and foraging areas. It may, however, be equally important to safeguard the interconnecting migratory corridors. The clustering of many individuals in space and time along migratory corridors could greatly increase the vulnerability of entire populations. The migratory path observed here may be representative of the migration route of all Pacific leatherbacks in the vicinity, and possibly in the entire Central American region. At the same time, such concentrations of turtles could facilitate protection of world stocks, simply by restricting potentially harmful activities within the spatial and temporal corridors.

The convergence of sea turtles along such constricted routes could have far-reaching ecological implications. It is possible that observed patterns of sea turtle migration are strongly influenced by distribution and availability of marine resources. Hence, by describing the routes of leatherbacks, we also may be depicting oceanographic features on a broader scale. If migratory routes are closely linked to mesoscale features or broader-scale environmental patterns, then current declines in sea turtle populations may reflect concurrently diminishing ocean resources.

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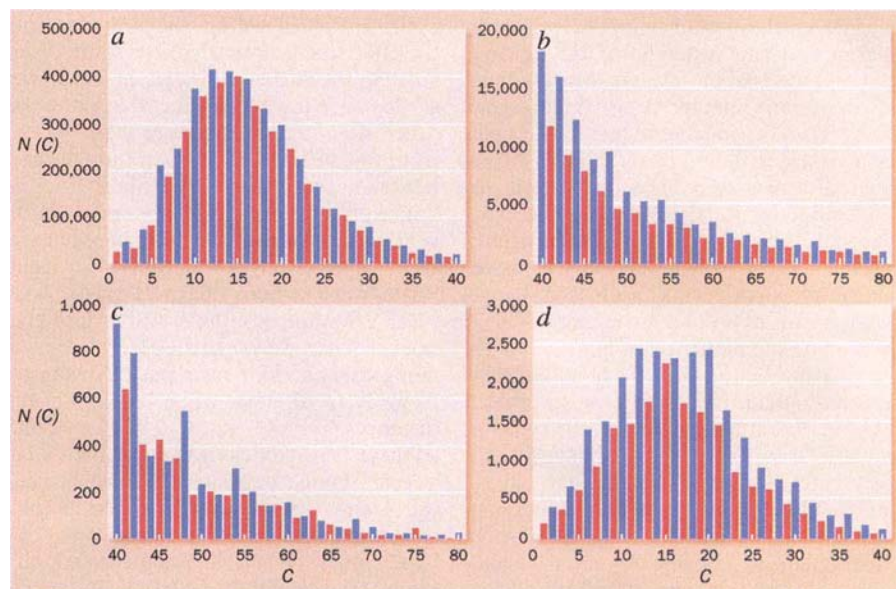
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Even-odd carbon atom disparity

SIR — We have stumbled on a striking feature of the composition of organic compounds, which, we believe, has not been noticed previously, and for which we have no simple explanation. In the sample of almost seven million organic compounds whose atomic compositions are listed in the Beilstein database¹, there is a



a, Frequency, $N(C)$, of occurrence of organic compounds in the Beilstein database as a function of the number, C , of carbon atoms in the molecule in the range $C=1$ to 40; b, same as a for the compositions with $C=40$ to 80; c, same as b but restricted to compounds isolated from natural sources; d, same as a but restricted to organic compounds listed in the CSD.

regular alternation in the frequency of occurrence of compounds with even and odd numbers of carbon atoms. There are more organic compounds with an even number of carbon atoms than with an odd number.

This alternation is discernible in the histogram (a in the figure), where it stands out against the strongly peaked distribution with its tip around $C=12$ to 16, and it becomes unmistakable once the distribution begins to flatten out at larger C values, for example in the range $C=40$ to 80 (b in the figure). A similar even-odd alternation is discernible in the subpopulation containing exclusively natural product compounds (c in the figure; ref. 1), although here it is not so pronounced. It is also found in various other smaller subpopulations, such as those in the *Handbook of Chemistry and Physics*² and in

catalogues of commercial supply houses, as well as in the collection of organic crystal structures listed in the Cambridge Structural Database (CSD) (d in the figure; ref. 3).

One can think of reasons why the CSD sample may be biased in favour of compounds with C even, for example, preference for centrosymmetrical molecules (about 12% of crystal structures in the CSD contain molecules located on inversion centres^{4,5}), but the reason for the preference for even carbon numbers in

the totality of organic compounds is not obvious. Could there be some fundamental underlying parity rule that is yet to be discovered?

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Scientific Correspondence

Scientific Correspondence is intended to provide a forum in which readers may raise points of a scientific character. Priority will be given to letters of fewer than 500 words and 10 references.

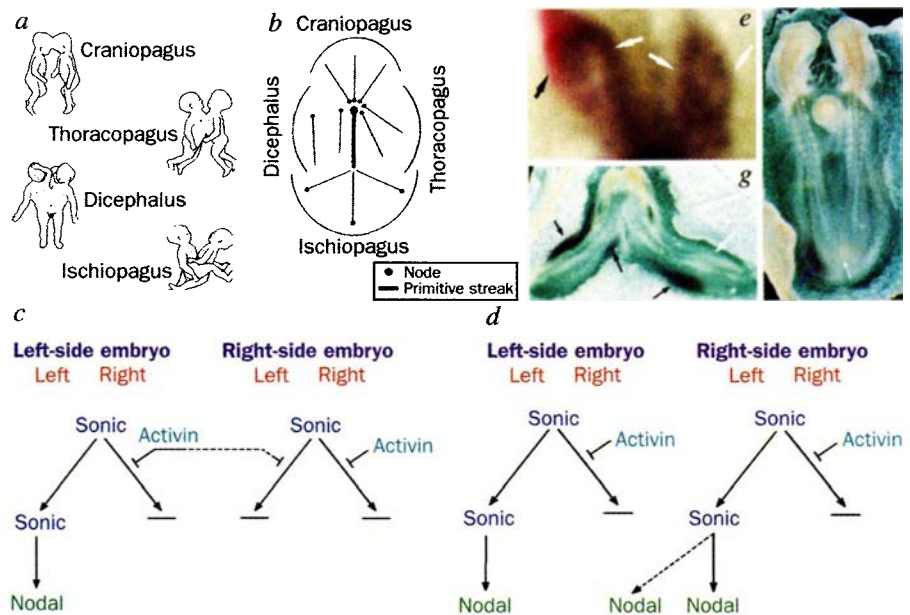
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Laterality defects in conjoined twins

SIR — Gene expression in twin chick embryos provides insight into the cause of the laterality defects in human conjoined twins^{1–3}. We surveyed 167 pairs of conjoined twins obtained locally and from the literature (details and references available from D. J. R. on request; e-mail: robertsd@helix.mgh.harvard.edu). The propensity towards laterality defects depends on the orientation of the conjoined twins (*a* in the figure). In almost half of the cases (33 of 69) where the twins were joined obliquely at the chest and/or abdomen (thoracopagi) or laterally at the chest (dicephali), one of the twins had reversal in heart situs; however none of the twins (of 98 studied) joined only at the head (craniopagi) or pelvis (ischiopagi) exhibited laterality defects. Strikingly (as noted in refs 1–3), when laterality defects are present, they occur most frequently in the twin on the right side in dicephalus (86%) and thoracopagus (71%) twins.

In the chick, a cascade of secreted signals during gastrulation regulates left–right asymmetry⁴. Activin, on the right side of the primitive streak, represses expression of the gene *Sonic hedgehog* (*Shh*) on the right. The remaining expression of *Shh* on the left is responsible for inducing *nodal* on the left, ultimately specifying heart situs. When twins arise from two parallel primitive streaks, there is the possibility of the activin produced on the right side of the left embryo inhibiting the expression of *Shh* in the left side of the right embryo (*c* in the figure). This would result in a normal left embryo, but the right embryo would have no expression of *Shh* in the node, and therefore no expression of *nodal*, and hence would have random heart situs⁴. Consistent with this hypothesis, we find that in spontaneous twin chick embryos with parallel primitive streaks, *Shh* (*e* in the figure) and *nodal* (*f*) are always expressed normally on the left of the left-side embryo, but never observed on either side of the right-side embryo.

If the twin primitive streaks form at an angle rather than being completely parallel, the two nodes become closer together as the streaks elongate. In some instances, the repression of *Shh* by activin will proceed normally because the streaks will initially be too far apart for the signals to diffuse. However, as the embryos elongate anteriorly, the *Shh* product (*Shh*) on the left side of the right embryo could cause *nodal* to be induced on the right side of the left embryo. In this case, the right embryo will be normal, but the left embryo will have double-sided *nodal* expression, leading to



Four classes of human conjoined twins (*a*, modified from ref. 11), have been suggested to arise from ectopic streaks (*b*, modified from refs 5–7). Parallel twin streaks (*c*) potentially allow activin cross-signalling to the right embryo. Such embryos hybridized to a *Shh* probe show normal *Shh* expression (magenta stain, black arrow in part *e*) in the left embryo, and a lack of expression (white arrows) in the right at embryonic stage 4–5 (2 of 2 cases examined). When hybridized to a *nodal* probe, such embryos show normal *nodal* expression (blue arrow, bottom in part *f*) in the left embryo and a lack of expression (white arrow, bottom) of *nodal* in the right embryo, which can result in inverted heart situs (black arrow) (2 of 2 cases examined). Oblique twin streaks that only become close as they extend (*d*) show normal *nodal* expression (*g*; black arrows, compare with white arrows) in the right embryo and double *nodal* expression (black arrows) in the left embryo (4 of 4 cases examined). The study was made possible by the assistance and cooperation of J. Tabin, S. Van Praagh, R. Van Praagh, M.-N. Westgate, P. Iyengar, J. Westerdahl, the Active Malformations Surveillance Program, the Departments of Pathology at Brigham and Women's Hospital and Boston's Children's Hospital, the Hensel family, and the many additional families contacted for information on family members, conjoined and separated.

randomization of its situs (*d* in the figure). This is precisely what we observed in obliquely oriented spontaneous twin embryos (*g*).

There have been several models of the origins of various classes of conjoined twins based on the relative orientation of their primitive streaks at gastrulation (*b* in the figure; refs 5–7). In craniopagi and ischiopagi twins, the primitive streaks are end-to-end, and signalling molecules to the side of one would not be expected to affect the other. However, dicephali and thoracopagi twins arise from streaks positioned adjacent to each other, potentially allowing cross-signalling. When primitive streaks are not completely parallel, which embryo has laterality defects will depend on their relative angle and distance apart. One would expect the left-side twin to have laterality defects in a higher proportion of thoracopagus conjoined twins than in dicephalus twins, as they result from primitive streaks oriented at a more oblique angle. This is indeed what is observed.

Activin, *Shh* and *nodal* normally act only on the side of the embryo where they are expressed, yet can apparently signal at a longer range between embryos. This suggests the presence of a barrier in the

midline of the embryo to confine each signal to its appropriate side. There is evidence that, in the amphibian *Xenopus*, midline structures do indeed facilitate regulation of situs⁸.

Although there are differences in the regulation of left–right asymmetry in birds and mammals, some aspects such as *nodal* expression are conserved^{9,10}. As such, the chick model provides the first coherent explanation for the laterality defects observed in conjoined twins.

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DNA binding by the ETS domain

SIR — The *ets* gene family encodes eukaryotic transcription factors that bind specific DNA sequences through the highly conserved, 85-residue ETS domain. The structures of two ETS-domain proteins, PU.1 and Ets-1, in complex with DNA, were described recently by crystallographic and by NMR spectroscopic methods, respectively^{1,2}. These reports were apparently contradictory, although an erratum to ref. 2 now resolves this controversy.

the DNA. However, the original NMR-based study positioned the HTH on DNA in an orientation rotated by nearly 180°, opposite to that seen in the crystal structure. The two structures also differed in the roles that helix H1 and the β -sheet play in DNA binding.

It seemed unlikely that the homologous DNA-binding domains of PU.1 and Ets-1 would bind DNA differently. Indeed, similar DNA contacts are ob-

of ethylation interference (phosphate numbers 2, 3, 5', 6', 7' and 8') are detected consistently in the analyses of ETS-domain proteins Ets-1, Fli-1 and GABP α (refs 3, 4; b in the figure). As exemplified by several previous structural studies (including *EcoRI*⁸ and λ repressor⁹), this technique identifies phosphates likely to be involved in DNA-protein interactions. Thus, the ethylation-interference data provide a signature pattern of contacts for evaluation of any model of ETS-domain-DNA interactions.

The pattern of phosphate contacts identified by ethylation interference implicates the PU.1 crystal structure and the revised Ets-1 NMR structure as the appropriate model for ETS-domain-DNA interactions. The figure compares the sites of ethylation interference with the phosphate contacts reported for the PU.1 and the original Ets-1 complexes. The pattern of backbone contacts identified in the crystal structure of PU.1 accounts for all predicted phosphate contacts¹. Lack of complete concordance is likely to reflect variation among ETS domains (see figure legend). Phosphate contacts implicated in the revised Ets-1 NMR structure are also similar to the sites of ethylation interference (ref. 2 erratum, Brookhaven protein data bank, accession number 2STT; M. Werner, G. M. Clore and A. M. Gronenborn, personal communication). These findings support the ETS-domain mode of DNA binding that involves both the β -sheet 'wing' and HTH motif. Furthermore, these analyses illustrate how structural and biochemical approaches can be used in combination to evaluate models of protein-DNA interactions.

Barbara J. Graves

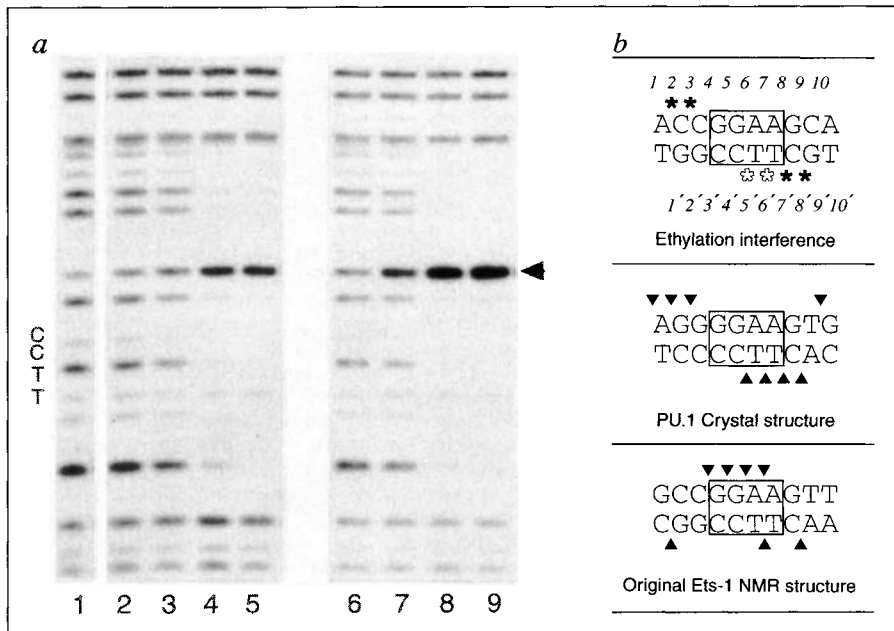
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Biochemical analyses distinguish between proposed models of ETS-domain-DNA binding. **a**, Similar modes of DNA binding of Ets-1 and PU.1 as detected by DNaseI footprints. DNaseI protection assays were performed with Ets-1 (lanes 2–5) and PU.1 (lanes 6–9); lane 1 displays the pattern of DNaseI cleavage in the absence of protein. Minimal fragments bearing the ETS domain were used (Ets-1, residues 331–440; PU.1, residues 167–271). Use of the high-affinity binding site SC1 (ref. 3) enabled this direct comparison between PU.1 and Ets-1 as this sequence 5'-ACCGGAAGCA-3' is recognized by many ETS-domain proteins⁴. Arrowhead, DNaseI hypersensitive site between two guanine residues on the TTCC strand. **b**, Phosphate contacts between ETS domain and DNA as implicated by ethylation-interference analysis^{3,4}, the crystal structure of PU.1-DNA (Fig. 2 of ref. 1), and the original NMR-based structure of Ets-1-DNA (Fig. 5 of ref. 2). The revised Ets-1 NMR structure shows contacts similar to those observed with PU.1. Coordinates identify the 5' phosphates of the 10 base pairs of each duplex; asterisks, sites of ethylation interference; arrowheads, phosphate contacts from structural studies. The contacts at phosphate 1 and 10 are made by PU.1 residues not conserved in other ETS domains. Phosphates 5' and 6' were not identified in the ethylation-interference analysis of Elf-1 (open asterisks)⁴. The lysine that contacts these positions in PU.1 is highly conserved within the family, but not present in Elf-1.

Our biochemical analyses of ETS-domain interactions with DNA (refs 3, 4 and work reported here) provide an independent resolution to this controversy and a verification of the current model of ETS-domain-DNA interactions.

The two structural studies^{1,2} confirm that the ETS domain is composed of three helices (H1, H2, H3) and a four-stranded, antiparallel β -sheet, placing the ETS-domain proteins in the winged helix-turn-helix (HTH) structural family^{5–7}. Further, in both structures the HTH (helices H2 and H3) recognizes the 5'-GGAA-3' consensus sequence in the major groove of

served in biochemical analyses of several ETS-domain proteins. In DNaseI footprinting studies, a hypersensitive site on the TTCC strand at a precise location is an invariant feature of ETS-domain-DNA interactions. Using a binding site recognized by many ETS-domain proteins, we show here that the DNaseI footprints of Ets-1 and PU.1 are identical (**a** in the figure). DNaseI footprints of other ETS-domain proteins, Fli-1, GABP α and Elf-1, are also the same⁴.

Ethylation-interference analyses also suggest a similar mode of DNA binding for ETS-domain proteins. Six major sites

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Cosmological wars

Hermann Bondi

Cosmology and Controversy: The Historical Development of Two Theories of the Universe. By Helge Kragh. Princeton University Press: 1996. Pp. 500. \$35, £27.50.

As one who has been intimately involved in the subject, I thoroughly appreciated reading this work in the history of science dealing with the evolution of cosmology. It centres on the period from the publication of the steady-state theory of the Universe in 1948 to its marginalization almost 20 years later, the 'Big Bang' theory being the chief alternative throughout. I am sure the book will be enjoyed by many, whether their main interest is indeed in cosmology, or in the interplay of personalities and the growing observational evidence, or in a piece of history in which failures of communication as well as friendships played an important part.

The book recounts in often riveting detail how scientific interest in the theory of cosmology was awakened by Einstein nearly 80 years ago and how almost simultaneously the observation of the redshifts of distant galaxies by Slipher (the importance of whose work is rightly acknowledged) ushered in much wider involvement in the subject. The scene is well described in which there was much theorizing by de Sitter, Friedmann, Eddington, Lemaître, Milne, Dirac, and many others, but few data. So the background is set against which in 1948 Gold and I, and Hoyle, proposed the steady-state theory of the expanding Universe.

There follows the core of the volume which tells how this theory gained only a few, but very significant, adherents, and attracted much opposition. The theory's vulnerability to observational tests (of which its originators rightly boasted) led to several alleged disproofs which were then themselves discredited until, after nearly two decades, accumulating evidence led to the theory being generally abandoned as a credible alternative to the ruling 'hot Big Bang' model. The author then recounts the aftermath in somewhat less detail.

Why do I recommend this book to a wide readership? First, because there are still many (not necessarily among those who peruse *Nature*) who think that disagreements are atypical of science. I often meet journalists who find it odd that there are differing scientific views on, say, environmental questions. Second, there are those, particularly common perhaps among sociologists of science, who decry

the crucial importance of empirical tests for the survival, or otherwise, of theories. Third, there are still a few left who believe that science advances relentlessly in a straight line. The actual zigzag path is beautifully illuminated in this book. For all



Bondi: proposed the steady-state theory of the expanding Universe.

such heresies it can serve as an excellent corrective.

Yet at the same time it must be admitted that philosophy has played a major role in the development of cosmology, especially perhaps in the period from 1920 to 1955. Differing philosophical, theological and ideological attitudes have made authors look in different directions. But this is not really peculiar to cosmology. In the entire scientific enterprise no part is as little guided by rational and mechanistic prescriptions as the choice of research topics and the approaches selected. The 'scientific method' cannot be put in a series of rules. Karl Popper expounded this point clearly, while stressing the supremacy of empirical disproof. Peter Medawar talked and wrote about it beautifully in *The Art of the Soluble*. There is little reason why cosmology should be different, although in the period in question the paucity of data naturally allowed for a wide variety of concepts.

In this connection there is an issue perhaps insufficiently stressed in Kragh's

book — that cosmology as an observationally accessible subject requires a time (and therefore length) scale. In a scale-free universe there can be no appreciation of whether any observation is cosmologically relevant or not, as one can have no knowledge of whether the region in question is significant or insignificant on the cosmic scale. The discovery of the redshift-distance relationship meant that such a scale became available through the inverse Hubble constant.

The fundamental importance of this feature was not perhaps appreciated at the time; now it is central to the ability to interpret observations in a cosmic context.

Note that, even before Slipher and Hubble, both the Einstein and the de Sitter models needed a length scale in the form of the cosmological constant.

As a principal actor on the scene described, I am full of admiration for the thoroughness of Kragh's historical researches. I must admit that I was astonished to find that it was possible to gather evidence, both written and oral, that enabled the developments to be described in such formidable detail. But my reference to detail should not frighten off potential readers: the text flows beautifully, smoothly and in places amusingly.

Yet it is a legitimate task for me as reviewer to state whether I regard this book as correct and fair to the steady-state theory and to my involvement in particular. My answer is a resounding 'yes'. On a few details (particularly perhaps dates) my recollections may differ slightly from Kragh's account but, frankly, I trust the author's researches better than my memory. Naturally there are some places where my emphasis would differ, but then this book is Kragh's, not mine. As is correctly stated, I have been out of the field since the late 1950s and so cannot bear witness to the accuracy of the description of later events to the same extent. But there is certainly no conflict between the book and my appreciation of what happened during this later period.

I should make two specific points, however. First, Kragh gives little emphasis to the philosophical point (often stressed by Gold and me) that in an evolving universe it requires an arbitrary assumption to select which features of present-day laboratory physics, notably the dimensionless numbers, depend on long-range interactions, and so change as the Universe changes, and which are true constants. The need to make such an arbitrary choice does not arise in the steady-state theory.

Second, it is my recollection that already by 1950 I had challenged the advocates of a changing universe to

produce some fossil remains of a supposedly different earlier state of the cosmos. Although I was blissfully unaware of the possibility of a cosmic background radiation, I was fully conscious of the threat to the steady-state theory posed by the then only very roughly known helium abundance. Kragh seems to have found no evidence of my having said this, so for once I must have been too quiet.

As regards fairness to others, I have already expressed my pleasure that Slipher is given full credit for his work. Equally I am delighted at the stress on Lemaître's contribution, which is not often mentioned today. Milne's time-scales are described, with perhaps an overemphasis on their supposedly conventional character. But I am a little uneasy that his seminal contribution of the definition of distance measurement by radar (well before the existence of radar became public knowledge) receives far less than its appropriate honour. This was the first time the unsatisfactory and misleading concept of the 'rigid rod' was dispensed with.

This volume can be thoroughly recommended to all interested in the evolution of cosmology and, indeed, more generally to all who like to see how improvements in observational technology affect the climate of opinion or, even more generally, how science develops. □

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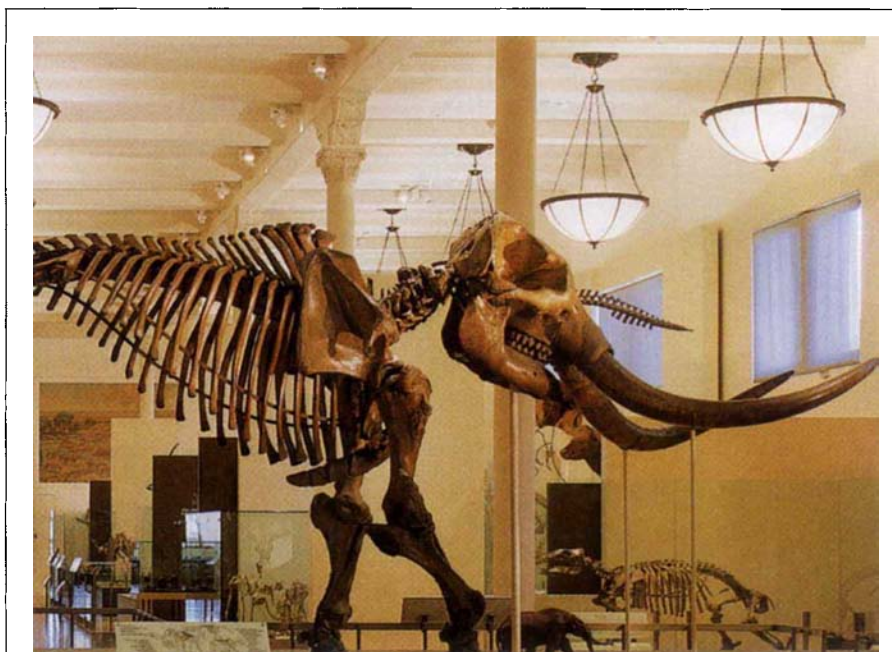
Spineless debates

Philippe Janvier

Before the Backbone: Views on the Origin of the Vertebrates. By Henry Gee. Chapman and Hall: 1996. Pp. 346. \$64.95, £35.

THE fact that man is a vertebrate, like all other animals with a backbone, is probably the main reason why most books on the origins of the vertebrates written since Lamarck's *Philosophie Zoologique* in 1809 have raised controversy. Early statements about vertebrate origins were generally derived from far-fetched comparisons with molluscs. Lamarck believed that the "considerable gap" between molluscs and vertebrates would be filled by animals that "remain to be discovered".

This gap was never filled and zoologists then imagined that the vertebrates derived from other segmented animals, the annelids and arthropods. Strangely, the detailed study of lancelets and sea-squirts and the discovery of their affinities with vertebrates did not make zoologists change their minds easily. These ideal 'liv-



FOLLOWING the main pathway through the fossil halls at the American Museum of Natural History in New York is like walking along the trunk of the vertebrate evolutionary tree. The 1.5 million visitors who take this route each year encounter this 11,000-year old mastodon, a fossil relative of elephants. These galleries have undergone a ten-year restoration directed by Lowell Dingus, who tells the story of the project, and describes the museum's extinct residents, in *Next of Kin: Great Fossils at the American Museum of Natural History*. Rizzoli, \$40 (hbk), \$24.95 (pbk).

ing ancestors' of vertebrates were instead regarded as 'degenerate' vertebrates. As zoology, anatomy and embryology progressed, more evidence was assembled to back up theories about vertebrate origins, yet the controversies remained heated.

Henry Gee tells an exciting tale of these debates in *Before the Backbone*. The book shows how scientists such as Patten, Gaskell, Garstang and Berrill have elaborated their theories, often in reaction to each others', and in the framework of a particular evolutionary process fashionable at the time (natural selection, recapitulation of ontogeny, pedomorphosis and so on). Gee also shows how, since the 1980s, phylogenies based on molecular sequences have added many characters to the search for vertebrate relationships, and he provides an up-to-date account of the influence of developmental genetics on questions of vertebrate origins and the patterning of the vertebrate head.

In addition to this excellent historical account, Gee gives a more scholarly description of the fossilized and Recent deuterostome groups involved in the debates about vertebrate origins. All the basic anatomical terms are explained with simple words and with illustrations, occasionally of items in his own kitchen — and toy box. He also gives a short introduction to cladistics, the methodological framework he uses throughout the book.

In his preface, Gee points out that the question of vertebrate origins has mainly been addressed on the basis of extant taxa.

How fossils can contribute to this question remains a problem, so he devotes nearly a third of his book to R. P. S. Jefferies's highly controversial 'calcichordate' theory, the only fossil-based theory about vertebrate origins proposed in this century. It rests on the idea that an ensemble of asymmetrical Palaeozoic fossils, classically regarded as echinoderms, are in fact a stem-group (colloquially known as 'calcichordates') from which arose independently the echinoderms, lancelets, sea-squirts and vertebrates. This theory implies that the calcitic skeleton has been lost several times and that the bilateral symmetry of vertebrates is a secondary feature.

The theory as well as its critics are objectively analysed, and its stimulating aspects are underlined. In particular, Gee shows how this theory raised the question of the asymmetry in various chordate groups (including some vertebrates), which even developmental genetics has difficulties in answering. Having a calcitic skeleton and gill slits, the 'calcichordates' also provide an example of the association of characteristics today regarded as unique to echinoderms and chordates, respectively.

Gee concludes with some general remarks, pointing out unanswered questions and new fields of investigation. He insists that most debates on vertebrate origins are based on a few living deuterostome groups — that is, on a depauperate biodiversity. This may influence phyloge-

netic analyses, because characters regarded as unique to a living group may well have had a much wider distribution, as possibly shown by the 'callichordates'.

The book reads extremely well, as would be expected from an author who is an assistant editor of *Nature* and a popular science writer. Each chapter is followed by a series of notes explaining technical terms in the main text and discussing certain points in more depth.

Before the Backbone is warmly recommended to professional biologists and palaeontologists, historians of science, lecturers, students, and even amateur zoologists and palaeontologists. It provides not only an up-to-date account of the debates on vertebrate origins, but also a critical assessment of the methods and aims of biologists and palaeontologists. □

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Measuring science revolutions

Mark Buchanan

Conversations with the Sphinx: Paradoxes in Physics. By Etienne Klein. *Souvenir*: 1996. Pp. 211. £18.99, \$22.95.

ALBERT Camus was probably right when he said that "All great deeds and all great thoughts have ridiculous beginnings". Had he been a scientist, he might have added that all great theories have paradoxical beginnings.

Such is the conclusion of Etienne Klein in his new book, which offers a philosophically light-hearted look at the significance of paradox in science. Paradox sometimes involves self-contradiction, as in "this sentence is false", a statement that ties itself in a self-referential knot. But Klein focuses on paradox of a different sort.

To a scientist, a paradox is a seemingly impossible conclusion that follows inescapably from a combination of unquestionable beliefs. And the emergence of paradox spells trouble. At the end of last century, for example, Lord Rayleigh showed that Maxwell's electrodynamics, coupled with classical statistical mechanics, requires all objects to glow with infinite brightness, an obvious absurdity.

Klein entertainingly describes a handful of examples, ranging from the paradoxical null result of the Michelson-Morley experiment, to the still problematic role of measurement in quantum mechanics. In each case, he shows that although paradox does indeed spell trou-

ble, it is precisely the discomfort that it engenders that directs the intellect towards deeper truth. As Klein puts it: "Paradox is truth standing on its head to get attention". The ultraviolet catastrophe forced Max Planck to question the notion that objects emit and absorb energy continuously. By formulating the idea of quantized energy exchange, he sidestepped the paradoxical infinity and ushered in the quantum era.

A paradox such as the "ultraviolet catastrophe" represents a kind of defect in the tightly woven network of interdependent ideas and beliefs that is science. Klein's thesis is that paradox focuses and amplifies intellectual stress in this network, and so sets the stage for upheaval, discovery and sometimes scientific revolution.

All this may seem rather obvious: science learns by making mistakes. In this sense, the book contributes no truly novel ideas, and sometimes reads like a condensed, although more animated, version of Thomas Kuhn's *The Structure of Scientific Revolutions*. Normal science, crises, scientific revolutions — they're all here, although sometimes in disguise.

Still, before reading this book, I thought of paradox as a sort of scientific equivalent of the pothole — an annoying but perhaps inevitable feature of the landscape. Klein establishes paradox in a more positive sense, as an essential mechanism by which science develops. This philosophical perspective is offered in the first half of the book, which is a well-written, carefully conceived essay. Klein clearly has an appetite for philosophy, and the text is littered with incisive quotations from Nietzsche, Kierkegaard, Bachelard and a score of others.

Unfortunately, the second half of the book, a hectic tour through the backyard of modern physics, is not so good. The sights are largely familiar: quantum non-locality, Schrödinger's cat, parity violation and so on — seven paradoxes treated in an all-too-brief 100 pages. Those who haven't already met these ideas will not understand much, and readers familiar with them will find nothing new.

That said, the ideas in this book may suggest a way to generalize and develop the Kuhnian perspective on science. For Kuhn, 'scientific revolutions' punctuate the normally slow and continuous process of scientific development. What is not clear, however, is how to identify a 'revolution' out of a background of normality. Can there be small revolutions? And could normal periods of science result not from the absence of revolution but from a barrage of small revolutions that together give the appearance of continuous development?

The latter dynamic strongly resembles processes that take place in other settings. For example, stress in the Earth's crust is released through earthquakes. Remark-

ably, the amount of energy released varies enormously — over many orders of magnitude — from one earthquake to another. The evolution of mechanical stress in the Earth is influenced not only by rare large earthquakes, but also by the much more frequent smaller ones. Might the evolution of science be similar?

Klein's notion of paradox can broadly be taken to refer not only to truly outstanding intellectual puzzles but also to many scientific problems of lesser significance. Some problems threaten to shake the foundations of all science, but most are less imposing, and their resolution affects beliefs and practices in a more limited way.

Seismological observations show that earthquakes that release an amount of energy E occur with a relative frequency proportional to $E^{-2/3}$. This is the Gutenberg-Richter law, and it implies, through its power-law form, that earthquakes have no typical size and that there is similarity in the mechanisms driving quakes of all sizes. If one could measure the size of a scientific revolution (perhaps through studies of citations?), and so estimate the magnitude of the rearrangement it entails, then one might similarly quantify the dynamics of scientific understanding. A study of the distribution of the sizes of episodes of scientific change would then reveal whether the Kuhnian dichotomy of normal and revolutionary is really true, or if science instead develops by way of revolutions of all sizes. Might there be an 'intellectual Gutenberg-Richter law'? □

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The dangers of Green dogma

Julian Morris

Green Backlash: Global Subversion of the Environment Movement. By Andrew Rowell. *Routledge*: 1996. Pp. 504. £45, \$65 (hbk), £12.99, \$18.95 (pbk).

DURING the late 1980s, the media, spurred by groups such as Greenpeace and Friends of the Earth, fed us a diet rich in polyunsaturated environmental scare stories. We were warned that greenhouse gas emissions would result in runaway global warming, causing sea levels to rise so much that London and New York would cease to be habitable. Chlorofluorocarbons would destroy the stratospheric ozone layer, resulting in a catastrophic rise in skin cancers. Persistent organic pollutants would cause genetic mutations, making fish and humans alike infertile.

The exaggerated nature of these claims

and the often extreme policy recommendations that followed — the British Green Party famously announced that the United Kingdom's population would have to fall by a third if it was to survive the next millennium — inevitably invited a backlash.

Andrew Rowell describes this reaction from the perspective of someone deeply entrenched in the environmental movement. The book is the result of a two-year investigation, commissioned by Greenpeace, into the activities of the "anti-environmental movement". Rowell claims this is a coalition of right-wing organizations, big business and governments, whose primary objective is to subvert the environmental movement.

That subversion is happening, argues Rowell, through a process of verbal subjugation, whereby the 'anti-environmentalists' refer to themselves as the 'true environmentalists', while calling the 'true' environmentalists religious fanatics, Communists, Nazis or elitists. To justify such name-calling, the 'anti-environmentalists' employ 'counter-scientists' and publish 'counter-science' books that dispute claims made by 'true' environmentalists.

Rowell is right to criticize those who make scientifically unjustified statements about the future state of the world, such as Edward Krug, who has written that "[if] global warming occurs to the extent that the doomsday models predict, it will be of great benefit to the world". Such statements imply a level of understanding of the global climate that we simply do not have at the moment.

But Rowell undermines his own valiant effort to condemn these nonscientific claims by littering the book with statements of dubious validity such as, "global warming is actually occurring and it is caused by man-made emissions", and meaningless babble, such as: "hardly any business activity is sustainable" and "truth has been stretched to its limits".

Most disturbing, perhaps, is Rowell's dogmatic view that alleged environmental problems, such as man-made global warming, should be seen as fact, not theory. That is true counter-science — it is contra-scientia, against knowledge, for it denies even the possibility that at some point an alternative theory might be found that would better explain the data. As Hans-Georg Gadamer has pointed out,

"the more honestly and rigorously science understands itself, the more mistrustful it has become toward all promises of unity and claims of final validity".

Scientific theories remain theories, rather than 'truths' or 'facts', because no theory has yet been found that perfectly fits all data. So the possibility always exists that an alternative, better, theory might be found.

These progressions in science from one theory or set of theories to another were described by Thomas Kuhn as shifts in the scientific community's paradigms. For Rowell, however, "paradigm shift means scapegoating the environmental movement [and] greenwashing by industry". That is to say, the attempt to redress the balance of the scientific debate by introducing alternative theories is merely an attempt to undermine the dogma of the 'true' environmental movement.

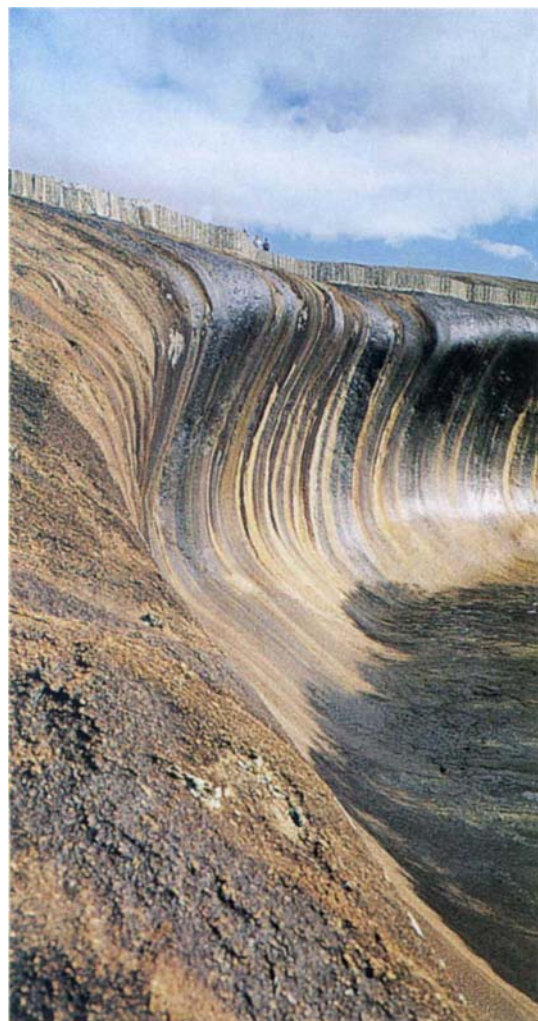
In Rowell's system, any shift in the public perception of environmental problems away from the conceptualization presented by "true" environmentalists would represent a "watering down of the scientific case for action". But, as Paul Feyerabend has pointed out, "theories cannot be justified and their excellence cannot be shown without reference to other theories". So what if the people proposing these alternative theories are funded by big business? It is the science that matters, not the paymasters. If an alternative theory fits the data better than the theory supported by the environmentalists, then this alternative theory should be preferred.

Karl Popper wrote that "from a biological or an evolutionary point of view science, or progress in science, may be regarded as a means used by the human species to adapt itself to the environment: to invade new environmental niches, and even to invent new environmental niches". But for science to progress, so that we may adapt ourselves to the future state of the world, scientists must be free to criticize each other's work.

The 'consensus' that Rowell demands would preclude criticism and hinder the creative process — it would kill off the very spirit of science.

What a tragedy it would be if, like Philolaus and Aristarchus, the originators of heliocentrism, the scientists currently proposing alternative explanations of environmental phenomena turn out to be correct but, because of the demand for consensus and the destruction of criticism, their ideas are lost, only to be rediscovered a millennium or so later by the inhabitants of a world impoverished by centuries of policies based on poor science. □

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EVERYBODY'S heard of Ayers Rock and the Great Barrier Reef but Australia has a rich variety of lesser-known yet equally fascinating natural landscapes. *Australia: A Continent Revealed* (New Holland, £29.99) explores these in more than 300 beautiful colour photographs. Wave Rock (left), near the town of Hyden, is one of Western Australia's most curious geological formations. This once-vertical granite wall, 100 metres long and 15 metres high, has been eroded into a smooth curving shape over the course of 3,000 million years. The rock is streaked with colourful vertical bands formed by rainwater reacting with chemicals within the granite. The Hyden area also contains Hippo's Yawn, Breakers and the Humps — weathered outcrops of granite known as inselbergs — and Aboriginal hand stencils on the walls of Mulkas Cave.

Pituitary lineage determination by the *Prophet of Pit-1* homeodomain factor defective in Ames dwarfism

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The gene apparently responsible for a heritable form of murine pituitary-dependent dwarfism (Ames dwarf, *df*) has been positionally cloned, identifying a novel, tissue-specific, *paired*-like homeodomain transcription factor, termed *Prophet of Pit-1* (*Prop-1*). The *df* phenotype results from an apparent failure of initial determination of the Pit-1 lineage required for production of growth hormone, prolactin or thyroid-stimulating hormone, resulting in dysmorphogenesis and failure to activate *Pit-1* gene expression. These results imply that a cascade of tissue-specific regulators is responsible for the determination and differentiation of specific cell lineages in pituitary organogenesis.

The anterior pituitary gland develops from a midline structure contiguous with the primordium of the ventral diencephalon^{1,2} and integrates signals from the peripheral and central nervous systems, regulating the production and secretion of critical regulatory hormones, including growth hormone, prolactin, thyroid-stimulating hormone, the gonadotropins and adrenocorticotrophic hormones by specific cell types (somatotropes, lactotropes, thyrotropes, gonadotropes and corticotropes, respectively)³. Contact of primordial stomadeal ectoderm and the neuroepithelium during head folding at embryonic day (e) 8.5 in the mouse⁴ results in the initial organ determination, coincident with the formation of Rathke's pouch. This developmental stage is characterized by expression of a series of homeodomain factors, including a LIM homeodomain factor (P-Lim/Lhx3)⁵⁻⁷ and an OTX-related homeodomain factor (P-OTX/Ptx)^{8,9}, restriction of expression to Rathke's pouch of a *paired*-like homeodomain factor, Rpx (Hesx-1)^{10,11}, and expression of the α -glycoprotein subunit^{9,12}. Following proliferation from a defined growth plate, different cell phenotypes arise in a distinct spatial and temporal fashion^{3,4}.

The Snell and Jackson allelic murine dwarf (*dw*, *dw*¹) mutations established that a POU domain gene, *Pit-1*, expressed in thyrotropes, somatotropes and lactotropes, located on chromosome 16 (refs 13, 14), is required for the initial expression of the genes encoding secreted hormonal products and receptors for releasing factors. *Pit-1* is later required for the continued expression of the *Pit-1* gene itself, and the proliferation and survival of these three cell types¹⁵⁻¹⁷. A mouse genetic defect, referred to as the Ames dwarf (*df*), is located on chromosome 11 (refs 18-20), is cell-autonomous²⁰, and results in a hypoplastic anterior pituitary similar to that of the Snell and Jackson dwarfs. In contrast to the complete absence of somatotropes, lactotropes and thyrotropes in the Snell mouse, the Ames mouse pituitary gland contains from ~1% (ref. 21) to 0.001% (ref. 22) of the normal

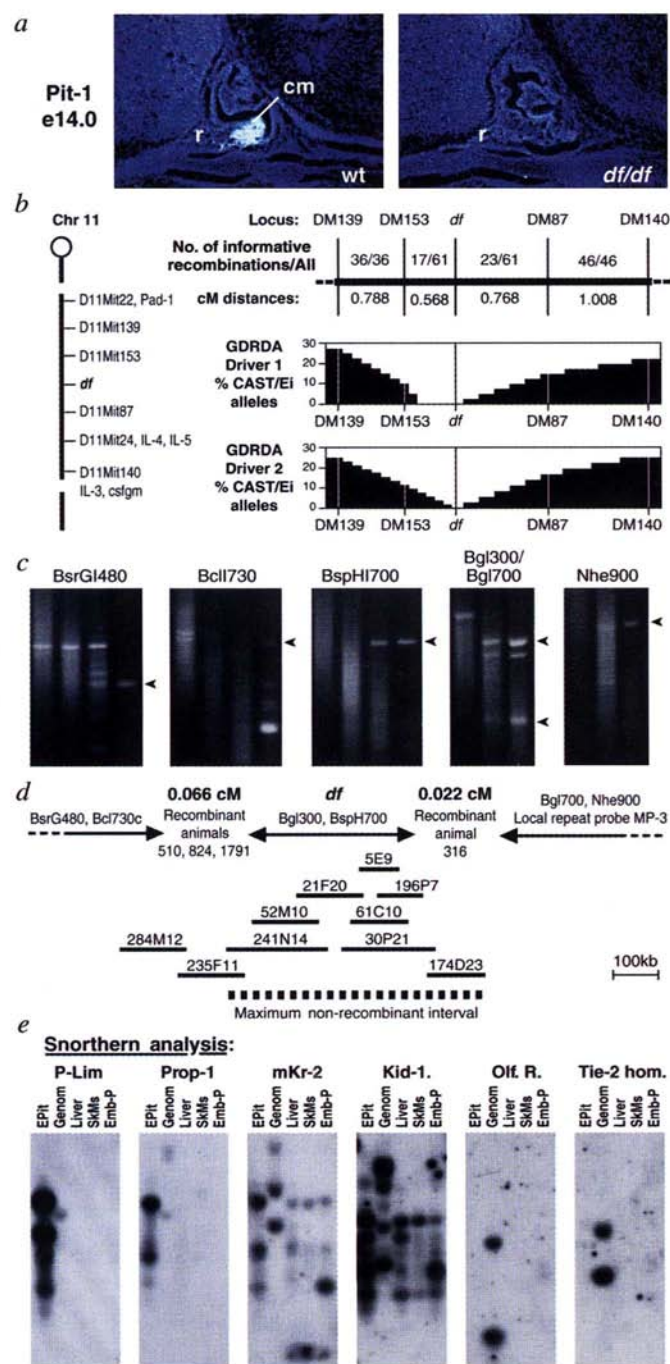
complement of somatotropes, as well as a few lactotropes and thyrotropes²¹. Failure to detect *Pit-1* transcripts later in ontogeny²² is consistent with the hypothesis that the Ames dwarf mutation is epistatic to *Pit-1*.

We now describe the application of genetically directed representational difference analysis (GDRDA)^{23,25} to achieve the positional cloning of a new gene, *Prophet of Pit-1* (*Prop-1*), a pituitary-specific, *paired*-like homeodomain factor. The *df* allele encodes a Ser → Pro mutation in the α 1 helix of the *Prop-1* homeodomain which results in significant functional impairment of *Prop-1*, and is postulated to cause a failure of determination of *Pit-1* lineages in the Ames dwarf.

Regions surrounding the *df* locus

On the basis of the failure of *Pit-1* gene activation in *df/df* pituitary glands²² throughout ontogeny (Fig. 1a), we started the positional cloning of the *df* genomic locus. The region surrounding the *df* locus was genetically mapped using a Cast/Ei × C57BL/6J intercross and a *Mus spretus* × C57BL/6J backcross (gifts from A. J. Lusis) to related CA-repeat markers²⁴ to the previously identified locations of the markers Pad-1 and the interleukin (IL) cluster (IL-3, IL-4 and IL-5)²⁰ (Fig. 1b). Four markers provided two proximal and two distal loci that could be used accurately to genotype progeny of an intercross between Cast/Ei and DF/B *df/df*. A total of 2,282 mice (equivalent to 4,564 independent meioses) were genotyped for the CA-repeat markers, and phenotypically characterized by size at 4–7 weeks (Fig. 1b). Pulsed-field gel Southern analysis indicated that the closest markers were separated by a minimum of one megabase (1Mb) (data not shown).

To obtain more closely linked markers, GDRDA^{23,25} was performed using two subsets of F₂ and F₃ *df/df* mice with local recombination events, targeting 0.5-centimorgan (cM) and 0.1-cM intervals surrounding *df* (Fig. 1b). After 3–4 rounds of



GDRDA using 26 different restriction enzymes for amplification in the polymerase chain reaction (PCR) of linked digests of genomic DNA (amplicons), six unique probes that closely flanked the *df* locus were obtained (Fig. 1c). Two probes mapped three recombination events centromeric to *df*; two probes mapped one recombination event telomeric to *df*; and two probes were non-recombinant with *df* (Fig. 1d). Using the estimate of 1.7 Mb cM⁻¹, the physical distance between the closest recombination events would be expected to be 30–50 kilobases (kb); however, pulsed-field genomic Southern analysis indicated a physical distance of ~400–600 kb, defining this region as a recombinational ‘cold spot’. Probes generated by GDRDA were used to obtain genomic clones in bacterial artificial chromosomes (BACs)²⁶. Genetic mapping of probes isolated from the ends of genomic clones by ‘vectorette’ PCR²⁷ revealed that the entire non-recombinant

FIG. 1 Generation by genetically directed representational differential analysis (GDRDA) of probes adjacent to the *df* locus. **a**, *In situ* hybridization using an antisense *Pit-1* probe reveals marked expression in the caudomedial (cm) portion of the wild type, but no detectable expression in the *df/df* gland, on e14.0; r, rostral tip. **b**, Comparison of the locations of (CA)_n repeat markers to previously described markers. Mapping of (CA)_n markers using a Cast/Ei × DF/B *df/df* intercross of 4,564 meioses. GDRDA drivers were created from 18 or 22 intercross *df/df* genotype F₂ or F₃ animals; subtraction of the Cast/Ei tester by Cast/Ei driver alleles targeted *df*. **c**, Agarose-gel electrophoresis of PCR products showing progressive enrichment of specific polymorphic alleles through 3–4 rounds of GDRDA using the indicated restriction enzymes (Bgl represents *Bgl*III). Arrows indicate visible products shown to be region-specific probes. **d**, Location of GDRDA probes relative to the closest informative recombination events to the *df* locus. Bgl-300 hybridizes to a unique non-recombinant locus, and BspH-700 hybridizes to two non-recombinant loci 10–50 kb apart. The BAC contig is shown, with sizes determined by pulsed-field gel analysis. **e**, Use of ‘Snorthern’ analysis as a secondary screen. DNA was generated using 4-base restriction enzyme site/oligo(dT) primers, linked, and amplified by PCR. Southern blots with each lane representing an equal mixture of *Ddel*, *Mbo*I and *Nla*III amplicons for the indicated tissues provide expression pattern representations. Epit, 1:1 mixture of amplicons from e13.5 and 15.5 pituitary cDNA syntheses; G, 4 µg *Dpn*II-digested mouse genomic DNA as a hybridization positive control; Liver and SKMs, adult liver and skeletal muscle amplicons; Emb-P, amplicons from an e15.5 embryo from which the pituitary was removed. P-Lim was used as a control to verify the predicted expression pattern of a pituitary-restricted gene. Probes for other indicated genes were obtained through cDNA selection or exon-trapping. Olf-R, a novel olfactory receptor; Tie-2 hom., a receptor tyrosine kinase most similar to that encoded by the mouse gene *Tie-2*.

interval of the *df* locus was encompassed by four overlapping BACs (Fig. 1d).

Characterization of the candidate gene

Exon trapping and complementary DNA selection^{26–30} were used to obtain the full complement of candidate genes within the interval. Candidate fragments were sequenced, assembled into contiguous DNA segments, physically mapped to the BAC ‘contig’, and shown to include regions from transcripts encoding three Krüppel-like zinc-finger factors, novel olfactory and glutamate receptors, a receptor tyrosine kinase homologue, and transcripts with no significant BLAST-search algorithm homologies. Over 15 fragments of each previously identified pituitary-expressed cDNA located within the contig, representing 80–90% of their complete sequences, were recovered, indicating that achieving saturation by these techniques was possible (data not shown).

To identify the candidate gene, we devised a technique to screen for transcripts selectively expressed in the embryonic pituitary. Amplicons of cDNA from e13.5–e15.5 mouse pituitaries and other tissues were generated using *Nla*III, *Mbo*I and *Dde*I restriction enzymes, then mixed as tissue-specific amplicons. Southern blots of the amplicons were hybridized with individual fragments from cDNA-selected and exon-trapped products. With this ‘Snorthern’ analysis, we identified only one transcript that was completely selectively expressed at high levels in the embryonic pituitary gland (Fig. 1e), with only trace amounts in the adult pituitary (data not shown). Twelve independent cDNA clones of the pituitary-specific candidate were obtained from an e13.5–e15.5 mouse pituitary λgt11 cDNA library. Sequencing of these clones revealed an open reading frame, which predicted a protein containing 223 amino acids (Fig. 2a). This predicted protein had highest homology with homeodomain factors of the *paired*-like family³¹, being most closely related to *Aristaless* and *Gooseberry*^{32–34}, but it did not contain an N-terminal *paired* domain or any other shared motifs outside the homeodomain (Fig. 2b). A CA-repeat located in the 5′ untranslated region (UTR) proved to be polymorphic, with (CA)₁₈ in the DF/B wild-type allele, (CA)₂₂ in the DF/B *df* allele, and approximately (CA)₁₂ in Cast/Ei, providing a readily typed polymorphism for analysis of DF/B genotypes in multiple-background strains.

df product and hypothalamic signalling

Analysis of the expression of the new homeodomain transcript during ontogeny revealed that its expression throughout development, like that of *Pit-1*, was detectable exclusively in the pituitary

gland. Although it was not detected, or only equivocally present, in the e9.5 Rathke's pouch (Fig. 3a), its expression was consistently detected by e10–e10.5, particularly over the dorsal portion of the gland. Expression was maximal by e12.0, with expression over the full caudomedial area in which *Pit-1* is first expressed on e13.5.

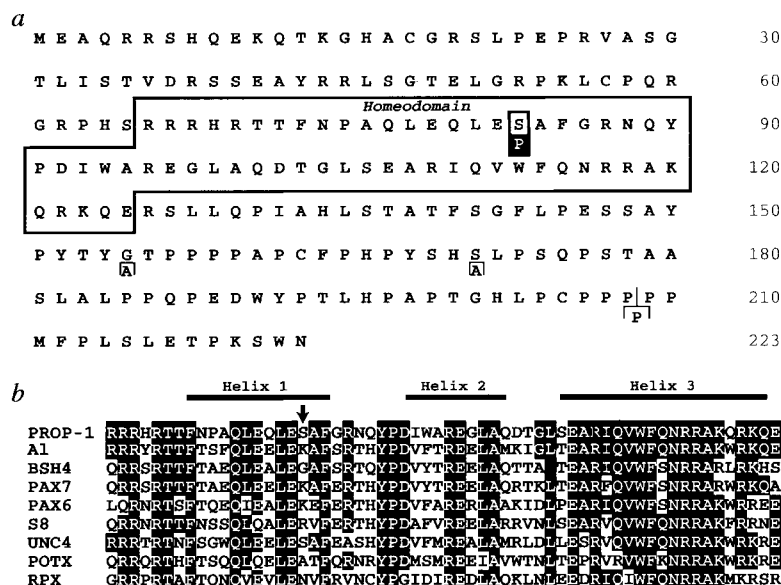
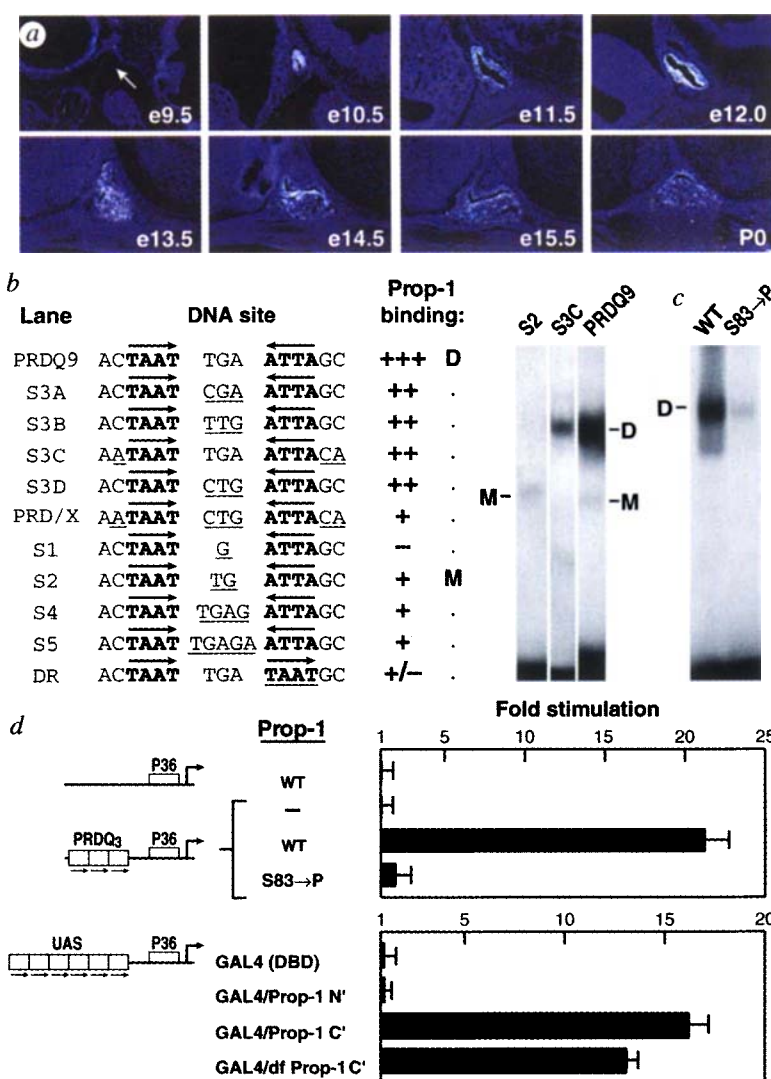


FIG. 2 Sequence of Prop-1 and comparison of its homeodomain with several known *paired*-like homeodomains. **a**, The predicted open reading frame in cDNA derived from C57BL/6 mice predicts a 223-amino-acid protein. Comparison to the DF/B *df/df* protein reveals a T → C transition in the sequence coding the first α -helix of the homeodomain, altering codon 83 from Ser to Pro, a Gly-to-Ala polymorphism at amino acid 155, and a Ser-to-Ala polymorphism at amino acid 171, caused by G → C and T → G transversions at nucleotide positions 464 and 508, respectively, and an insertion of a proline codon (ACC) between amino-acid positions 208 and 209. Also present is a polymorphic (CA) repeat in the 5' untranslated region (data not shown). The *df/df* sequence was confirmed by sequencing of 5 individuals; the wild-type (WT) sequence of these regions from six different strains was determined by PCR. **b**, Comparison of the Prop-1 amino-acid sequence to that of other *paired* or *prd*-like homeodomain factors. Arrow indicates the position of the *df* mutation in the first α -helix; Al is Aristaeless.

FIG. 3 Characterization of *Prop-1* gene expression and identification of a functional defect in the *df* factor. **a**, Ontogeny of *Prop-1* expression in the developing pituitary gland, with detectable expression by e10–e10.5 and peak expression at e12.0. **b**, Identification of DNA-binding sites for Prop-1 and impaired binding of *df* Prop-1. The consensus site of the *paired* homeodomain with a Q₉ in the third α -helix (PRDQ₉) represents the highest-affinity site. Other sites altering spacing, flanking nucleotides or orientation of TAAT core motifs were tested; representative gel retardation assays are shown. M, monomer; D, dimer. **c**, The *df* protein, or an S83P-mutant Prop-1, consistently gave an eightfold reduction in binding affinity to the PRDQ₉ DNA site. **d**, Prop-1 effectively transactivated a reporter containing three PRDQ₉ DNA binding sites fused to a minimal promoter (PRDQ₃ P36-luciferase), whereas Prop-1 S83P and *df* Prop-1 were impaired, even at high plasmid levels. The GAL4 DNA-binding domain (amino acids 1–147) fused to the N-terminal (amino acids 1–65) or C-terminal (amino acids 126–223) region of Prop-1 revealed the comparable ability of the wild-type (WT) or of Prop-1 C terminus to promote transactivation on promoters harbouring multimerized UAS sites ((UAS)₆ P36-luciferase).



After e14.5, *Prop-1* expression is markedly decreased. Because our cloned cDNA represented the only embryonic pituitary-specific transcript in the interval and had a temporal expression pattern consistent with the ontogeny of the *df* defect, argued strongly that alternations in this gene might underlie the *df* mutation. Because the *df* mutation appears severely to impair the appearance of Pit-1 lineages, the gene encoding the new paired-like homeodomain factor is referred to as *Prophet of Pit-1* (*Prop-1*).

Impairment of the *df* gene product

Although there was no evidence of rearrangement at the *df* locus, sequencing the entire candidate gene isolated from a λ genomic DNA library created from a single DF/B *df/df* individual revealed four alterations from the initially cloned cDNAs. A mutation (T $\rightarrow$ C) in the first α -helix of the homeodomain (Fig. 2a), altering a serine at position 83 to a proline (S83P), was present in the cloned Ames DNA and in genomic DNA from five additional *df/df* individuals, but not in DNA isolated from six different mouse strains. Of more than 400 known homeodomain factors³¹, not a single one has a proline in this position, consistent with the hypothesis that this mutation is not favourable for homeodomain function. This mutation would potentially disrupt the first α -helix of the homeodomain and its structure. Three other altered amino acids, G155A, S171A and an insertion of a proline residue at 208, are presumed to have predated the *df* mutation, based on assessment of function, as presented below.

Paired-class homeodomains bind as dimers to cognate DNA sites that are generally configured as TAAT palindromes spaced by 2–3 base-pairs (bp)^{35,36}. Because *Prop-1* contains a glutamine (Q) at position 50 in the homeodomain, the *paired* S50Q consensus binding site identified by random site selection³⁵ was tested,

revealing that *Prop-1* bound cooperatively as a dimer to this palindromic site. Alterations in flanking nucleotides, spacing between or orientation of core binding motifs markedly impaired dimer binding (Fig. 3b). *Prop-1* protein harbouring the S83P mutation was significantly impaired in DNA binding, with a 6–8-fold lower affinity than wild-type *Prop-1* on a high-affinity site (Fig. 3c), consistent with this being the critical mutation. Assessment of function by co-transfection of reporter with wild-type, or S83P *Prop-1* in heterologous cell lines showed that the mutation results in a marked decrease in efficacy of transcriptional activation (Fig. 3d). The C-terminal, but not the N-terminal, portion of *Prop-1* transferred the transactivation function to a GAL4 DNA-binding domain (Fig. 3d), with almost equivalent activity of the *df* *Prop-1* C terminus (Fig. 3d). The consequences of the S83P mutation are thus compatible with the marked, but not complete, loss of Pit-1 lineage cells in the *df/df* mouse; hence, the mutation of *Prop-1* appears to represent the aetiology of the *df/df* phenotype.

Developmental defects in *df/df* pituitary

No abnormalities were detected in the *df/df* pituitary gland up to e12, with a normal growth plate and appearance of rostral tip thyrotrope, corticotrope and gonadotrope cell types; however, a striking dysmorphogenesis becomes apparent at e13–e13.5, involving an expansion in the population of central plate cuboidal cells, with a marked linear expansion of the luminal structure (Fig. 4a). In parallel, there was a failure to accumulate the caudomedial glandular cells, in which Pit-1 is normally expressed. In the mature gland, there are a few α -melanocyte-stimulating hormone (α -MSH)-expressing cells abnormally present in the anterior lobe (data not shown). Several transcripts encoding homeodomain factors that are normally expressed on initial contact of primordial pituitary with the neuroepithelium on e8.5, including P-OTX/Ptx and P-Lim/Lhx3, gave normal patterns and levels of expression in *df/df* mice; similarly, there were no significant differences in the expression of *Six3*, *Pax-6* or *Msx-1* transcripts^{37–39}; however, these transcripts were also detected in the distorted periluminal cells, at levels now actually above those in the developing posterior lobe (Fig. 4b, and data not shown). Additionally, the expression of a series of homeodomain factors continued beyond their normal time of expression in the *df/df* pituitary gland. The *Prop-1* transcript was comparably expressed, and in as many cells, in *df/df* as in wild-type pituitaries, from e10.5–e15.5, but there was prolonged expression in the *df/df* gland (Fig. 5). Expression of *Rpx*, a *paired*-like homeodomain factor that is expressed in the prosencephalic plate before becoming restricted to Rathke's pouch at e8.5 (ref. 10), is normally decreased by e13 (ref. 10), becoming undetectable by e14–e15.5 in the wild-type gland (Fig. 5). In the *df/df* gland, however, *Rpx* was still readily detectable in periluminal cells up to e14.5, but was undetectable by e17.5 (Fig. 5). There was a similar prolongation of pituitary expression of the POU-domain factor Brn-4, normally only minimally detectable by e15.5 (Fig. 5). Conversely, the second wave of neuronatin expression⁴⁰, associated with the successive appearance of mature pituitary cell types, failed to occur on e14.5–e15.5 (Fig. 5) in the *df/df* pituitary. Together, these data are most consistent with a model in which there is a failure of determination of the Pit-1 lineages in the *df/df* mouse, with continued expression of a series of markers characteristic of the precursor cell.

Combined homeodomain codes

Because *Rpx* is apparently co-expressed with *Prop-1* from e10.5 to e13.0–e13.5 but decreases much earlier than *Prop-1* expression, we tested the efficacy of *Rpx* on *Prop-1* response elements and found that *Prop-1* and *Rpx* bound as heterodimers on cognate palindromic DNA-binding sites (Fig. 6a). Transfection studies using *Prop-1*-dependent promoters revealed that *Rpx* was ineffective at activating *Prop-1* DNA-response elements and interfered with *Prop-1*-dependent activation (Fig. 6a), indicating that these factors are functionally distinct and potentially antagonistic.

Because the failure of Pit-1 activation in the *df/df* mouse could

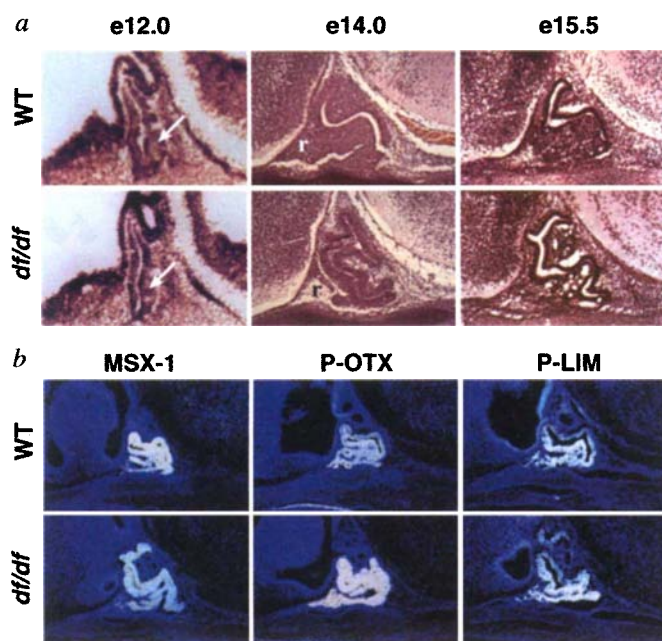


FIG. 4 Developmental defects in ontogeny of the *df/df* anterior pituitary. a, Haematoxylin-and-eosin staining of paraffin sections of WT and *df/df* pituitary gland from e12.0–e15.5, revealing normal morphology on e12.0, with subsequent distortion and expansion of the cuboidal cell layer surrounding the lumen and failure to generate the caudomedial, acidophilic granular cellular population. The white arrow indicates the pituitary gland at e12.0; at e14.0, 'r' indicates the location of the rostral tip. In the mature *df/df* gland, only a rare growth-hormone-positive cell was observed (~20 per gland); no TSH- β or prolactin-positive cells were detected, and the appearance of α -MSH cells in the anterior pituitary was atypical (data not shown). b, Levels of *P-Lim/Lhx3* and *POTX/Ptx* and *Msx-1* transcripts, as detected by *in situ* hybridization, were apparently unaltered in the *df/df* gland from e10.5–e15.5 (data from e14.5 shown).

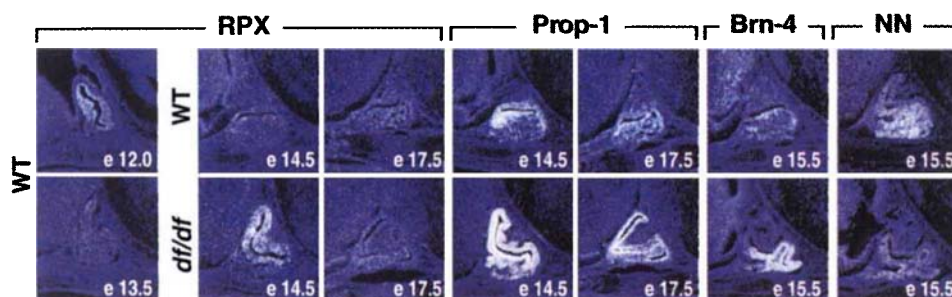
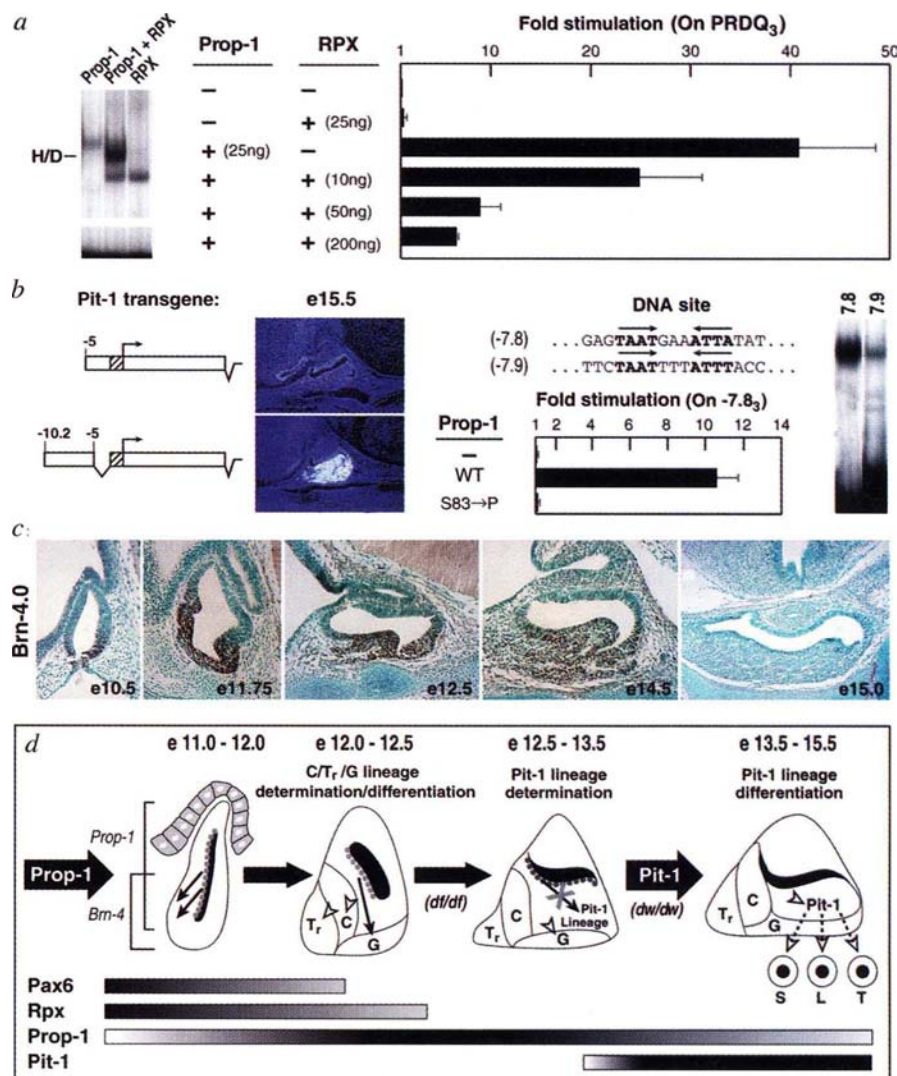


FIG. 5 Altered temporal patterns of factor expression in the *df/df* pituitary. Ontogeny of expression of *Rpx*, *Prop-1* and *Brn-4* transcripts in the *df/df* mouse compared with wild type. Each of these transcripts was expressed over an abnormally long period, as seen from examples on e14.5 or e15.5, but all were undetectable by e17.5 (*Brn-4* on e17.5, not shown). The loss of the caudomedial cell populations is further indicated by the failure of the second phase of neuronatin (NN) gene expression on e14.5.

FIG. 6 Model of *Prop-1* action in determining *Pit-1* lineages. *a*, *Prop-1* can heterodimerize with *Rpx*. 35 S-labelled *Prop-1* proteins, generated by transcription and translation in reticulocyte lysates, were used with a radiolabelled oligonucleotide representing the PRDQ₃ consensus site; at limiting concentration, *Prop-1*/*Rpx* heterodimers (H/D) were favoured. Results from co-transfection with a PRDQ₃ P36-luciferase reporter in HeLa cells using the indicated amount of *Prop-1* and *Rpx* expression plasmids were similar in three independent experiments. *b*, The -10 to -5 kb 5'-flanking sequences of the *Pit-1* gene conferred early activation function in transgenic mice. *In situ* hybridization at e15 revealed high levels of transgenic expression with the -10 to -5 kb construct, but no detectable expression with the -5 kb construct (three founders each). Two of the series of potential *Prop-1* sites present in the early enhancer region (-7.8 kb, -7.9 kb) bind *Prop-1* by electrophoretic mobility gel shift assay. *Prop-1*, but not *Prop-1* S83P, effectively transactivates promoters under the -7.8 element by cotransfection assay. *c*, Asymmetric expression of *Brn-4* at several stages of pituitary ontogeny. Data are similar in *Brn-4* *in situ* hybridization. *Msx-1* transcripts exhibit an early rostral to caudal gradient of expression (data not shown). *d*, Model of determination and differentiation events in pituitary ontogeny. The determination of corticotrope (C), rostral tip thyrotrope (Tr) and gonadotrope (G) cell phenotypes between e8.5–e11.5, and their subsequent differentiation occur before determination of the *Pit-1* lineages on $\sim$ e12.5–13.0, with *Pit-1* initially detected on e13.5. Determination/migration is denoted by solid arrows, differentiation by open arrowheads. The *Prop-1* mutation in *df/df* results in a failure of determination of the *Pit-1* lineages (e12.5–e13.0). X, determination block in *df/df* mouse. *Pit-1* gene activation and initial differentiation is followed by the appearance of three distinct cell types: the somatotrope (S), lactotrope (L) and thyrotrope (T) cell types.



be either a direct result of the defect, or a secondary outcome of a block in the determination of the *Pit-1* lineages, we investigated whether there were cognate *Prop-1* DNA-binding sites in regions of the *Pit-1* gene required for initial activation. Other data (G. E. DiMattia *et al.*, unpublished results) have revealed that the enhancer required for *Pit-1* autoregulation¹⁷ cannot stimulate initial pituitary-specific activation. As shown in Fig. 6b, a fragment encompassing -5 to -10 kb of *Pit-1* flanking information gives strong initial transgene activation, consistent with the presence of a distinct early *Pit-1* gene enhancer. Two of the putative *Prop-1* dimeric DNA sites in the -5 to -10 kb early enhancer bound *Prop-1*, one with high affinity (Fig. 6b). These sites served as response elements in transient co-transfection assays for wild-type, but not S83P, *Prop-1*; however, the -10 to -5 kb fragment

was not activated. Thus, if *Prop-1* itself has a direct function in the initial activation of the *Pit-1* gene early enhancer, other factor(s) and/or signals must be required.

Because of the decreased ventral expression of *Prop-1* at early developmental times (e10.5; Fig. 3a), we examined whether pituitary transcriptional regulators that might be involved in determination events were expressed in stratified patterns. *In situ* hybridization and analysis with specific antisera⁴¹ revealed that *Brn-4* was first expressed at the ventral tip of Rathke's pouch on e10, expanding to a distribution over the entire ventral portion, and finally limited to the region of *Prop-1* expression and subsequent activation of *Pit-1* (Fig. 6c). *Msx-1* transcripts^{38,39} were initially expressed in a rostral-to-caudal gradient, and *Six3* (ref. 37) ultimately was expressed preferentially in the intermediate

lobe (data not shown). The observation that several homeodomain factors, which are expressed either in highly restricted patterns or only in the nascent pituitary gland, exhibit zones of differential qualitative or quantitative expression, suggests the possibility of combinatorial codes of cell-type specification.

Discussion

Identification of the *df* mutation as a partial loss of function of a tissue-specific *paired*-like homeodomain transcription factor, causing a specific defect in determination of the Pit-1 lineages in the *df/df* pituitary gland, has provided insight into the molecular mechanisms that underlie the appearance of specific cell phenotypes during pituitary organogenesis. Cloning of the *Prop-1* gene represents an initial use of genetically directed representational difference analysis^{23,25} to solve a genetic defect.

The *df* defect is evident at e13.0–e13.5, as recently divided cells that would populate the caudomedial region in the developing gland fail to leave the growth plate, causing a dramatic dysmorphogenesis. There is an associated general loss of *Pit-1* gene activation, with failure of progression to morphologically mature secretory cells. As a consequence of the *df* defect, the expression of a number of transcription factors, including *Brn-4*, *Rpx* and *Prop-1* itself, becomes temporally extended. In contrast, the second phase of expression of the calbindin-related protein, *neuronatin*⁴⁰, which is normally highly re-expressed as mature cells are formed, fails to occur after e13.5 in the *df/df* gland, providing further evidence of the failure of determination of the newly divided cells after e13.5 in this defect. In contrast, the first three cell lineages determined on e11–e13.0 in the pituitary—rostral tip thyrotropes, corticotropes and gonadotropes—are unaffected, indicating that *Prop-1* is not required for the cell-type determination events of the initial three cell lineages.

The ultimate result of the *df* mutation is the severe depletion of the three Pit-1-dependent cell types. The ineffective activation of *Pit-1* in the *df/df* mutant could reflect either a failure of Pit-1 lineage determination and/or a potentially direct role for *Prop-1* in initial activation of the *Pit-1* early enhancer. Indeed, the switch of *Pit-1* gene expression to an autoregulatory mechanism¹⁷ occurs at the same time as the sudden drop in *Prop-1* transcripts. Because *Prop-1* alone fails clearly to activate the specific enhancer required for initial *Pit-1* gene activation, either additional factors or signals are required, or the effects of *Prop-1* are indirect.

Considered with the known roles of Pit-1 and *Lhx-3/P-Lim*, and the presumptive roles of P-OTX/Ptx and *Rpx/Hesx-1* (refs 8–11, 13, 15–17), the identification of *Prop-1* further defines the operation of a cascade of tissue-specific homeodomain factors in the initial organ and cell determination and subsequent differentiation of specific pituitary cell phenotypes (Fig. 6d). We have previously demonstrated interactions between pituitary homeodomains, including P-Lim, P-OTX, and Pit-1 (refs 5, 9), and have shown here that *Prop-1* and *Rpx* can bind cognate DNA elements as heterodimers, inhibiting *Prop-1*-dependent activation. The presence of *Rpx* until e12.5–e13.5 could serve to inhibit *Prop-1* actions earlier in pituitary development, thereby temporally delimiting the actions of *Prop-1* to determination of the Pit-1 lineage. The decreased affinity shown by the S83P mutation could be significant at physiological concentrations in light of the preference of *Prop-1* for dimeric binding sites, and the cooperative binding evidenced by close family members³⁵. We propose that *Prop-1* is one of a specific complement of interactive/heterodimeric partners, expressed between e10 and e13.5, which in combination dictate the patterns of gene activation and repression that underlie the determination of specific pituitary cell phenotypes.

Developmental events in pituitary and neuronal cell-type determination seem to be analogous in their patterns of cell-type appearance, with the pituitary following 'outside in' developmental events characteristic of the generation of nuclei in the central nervous system^{42–44}. We suggest that the lumen of the pituitary functions as a pituitary 'ventricle', and the adjacent growth plate as a pituitary 'ventricular zone'. The observation that both tissue-

specific and tissue-restricted homeodomain factors can be expressed in distinct spatial and temporal patterns in the critical period of pituitary organ and cell-type determination, between e8.5 and e13.5, is analogous to the model of compartmentalized development of rhombomeres dictated by *Hox* genes⁴⁴, and to proposed events in the prosencephalon⁴⁵. We suggest that these patterns establish zones in which successive waves of recently divided cells are determined as they leave the pituitary ventricular zone between e11.5–e13.5, developing into rostral tip thyrotropes, corticotropes, gonadotropes, and, finally, the precursors of the three Pit-1 lineages (Fig. 6d). In this regard, specific patterns of temporal and spatial restriction (for example, *Rpx*, *Brn4*), as well as regulation of gene activation by extrinsic and intrinsic signals, are also likely to be critical in cell-type specification.

Prop-1 may be an example of a tissue-specific determining factor that is required in organogenesis for the asymmetric cell division that generates a specific cell lineage. In light of the presumptive roles of *paired*-like homeodomains in other tissues, such as muscle and the branchial arch⁴⁶, embryonically distinct tissues may use parallel mechanisms in cell-type determination. □

Methods

Intercross and genetically directed representational difference analysis (GDRDA). Investigation of the simple sequence-repeat markers for polymorphisms was performed using PCR amplification with flanking oligonucleotides (D11Mit sequences²⁴; IL-3 and IL-5⁴⁷). The Cast/Ei × DF/B *df/df* breedings were achieved either through crossing Cast/Ei females to DF/B *df/df* males made fertile by thyroid supplementation, or Cast/Ei males were crossed to DF/B *df/df* ovarian transplants in SCID mice (Jackson Laboratories) F₁ mice and non-recombinant heterozygous mice of the F₂ and successive generations were intercrossed, generating 2,282 mice representing 4,564 independent meioses that were typed phenotypically and with D11Mit139, D11Mit163, D11Mit87 and D11Mit140. The GDRDA^{23,25} subtraction technique was applied using 26 enzymes with Cast/Ei genomic DNA as tester and two pools of genomic DNAs from Cast/Ei × DF/B *df/df* intercross F₂ and F₃ *df/df* animals as drivers. GDRDA was performed for 3–4 rounds of subtractive hybridization, kinetic enrichment and PCR. All regions except the immediate vicinity of *df* have Cast/Ei alleles in the driver, and are subtracted. GDRDA products were subcloned into pBKS (Stratagene), sequenced, and genetically mapped using the Cast/Ei × DF/B *df/df* intercross. An overlapping contig was generated from BAC (Research Genetics) and YAC (from S. Tilghman and H. Lehrach) clones, screened either by PCR using CA-repeat markers or through hybridization of probes obtained by GDRDA, or BAC and YAC and probes obtained through 'vectorette' PCR. MP is a 136-bp *Nla*III fragment of the Tfl vectorette centromeric side end clone of the D11Mit153 containing Whitehead superpool YAC B12.10B, hybridizing to 4 loci between D11Mit153 and D11Mit140.

Exon trapping and gene selection techniques. Exon trapping was done using the pSP3b vector²⁶. Two cDNA selection techniques were used to identify candidate genes. The first used filter-bound BAC/YAC DNA²⁹ and very high-stringency washes including an additional 5 min prewash step of water at 95.0 °C before hybridization, using double-stranded cDNA prepared from a mixture of adult pituitary and GHFT-1 (ref. 48) mRNA. DNA for selection was prepared from amplified vectorette linker digests of cDNA restricted with the enzymes *Rsa*I and *Alu*I (ref. 32). In the second selection technique, cDNA was prepared from 372 microdissected e13.5–e15.5 pituitary glands, using as primer oligo(dT) with an *Mbol*I, *Dde*I or *Nla*III site four bases from the 5' end. Enzyme-specific digestion, followed by linker ligation and PCR amplification provided cDNA used for hybridization to biotinylated BAC DNA, as described³⁵. For the second round of the cDNA selection procedure, a kinetic enrichment step was introduced by using 1% of the previous concentrations of cDNA and BAC DNA. DNA of subcloned products in pBKS was sequenced using an ABI 377A automated sequencer (Perkin-Elmer).

cDNA and genomic libraries. A cDNA library was generated in λ gt11 from 310 microdissected e13.5 to e15.5 pituitary glands with 1.5-mm diameter surrounding tissue, generating 1.2×10^7 independent clones. A DF/B *df/df* genomic library was generated in λ EMBL3 SP6/T7 (Clontech). All products of site-directed mutagenesis were verified by automated sequencing of the complete coding region for each construction.

In situ hybridization and immunohistochemistry. Isolation, fixation and hybridization with ³⁵S-labelled antisense RNA probes and exposure were all as previously described⁷. Embryos were genotyped using the four local CA-repeat polymorphic markers; recombinant embryos were not used. For immunohistochemistry, a 1:500 dilution of *Brn-4* rabbit antiserum⁴¹ was used, and donkey anti-rabbit IgG conjugated with horseradish peroxidase (Amersham) was used at a dilution of 1:100. Peroxidase activity was visualized with DAB/metal enhancer (Pierce; Rockford, IL).

Transfections and DNA-binding assays. Transfections and luciferase assays

were done in HeLa cells as described⁴⁷, using 1 µg luciferase reporter, 10–50 ng CMX expression vectors, and 1 µg pRSV-βGal as an internal control for differences in transfection efficiencies. Transfection data are expressed relative to luciferase activity of PRDQ₃ P36-luciferase, (7.8)₃ P36-luciferase, or (UAS)₆ P36-luciferase, which are defined as one. DNA binding was assayed using synthesized oligonucleotides phosphorylated with [γ -³²P]dATP, annealed, and gel-purified. Binding reactions and electrophoresis were done as described⁴⁷, using native Prop-1 and mutated forms prepared as reticulocyte lysate *in vitro* translations.

Additional details related to Methods are available as Supplementary Information.

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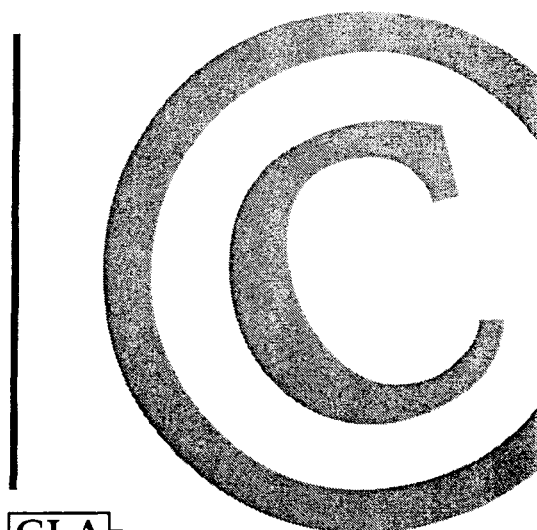
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Detection of H_3^+ in interstellar space

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THE H_3^+ ion is widely believed to play an important role in interstellar chemistry, by initiating the chains of reactions that lead to the production of many of the complex molecular species observed in the interstellar medium¹⁻⁵. The presence of H_3^+ in the interstellar medium was first suggested⁶ in 1961, and its infrared spectrum was measured⁷ in the laboratory in 1980. But attempts⁸⁻¹¹ to detect it in interstellar space have hitherto proved unsuccessful. Here we report the detection of H_3^+ absorption in the spectra of two molecular clouds. Although the present results do not permit an accurate determination of the H_3^+ abundances, these ions appear nevertheless to be present in sufficient quantities to drive much of the chemistry in molecular clouds. It should soon be possible to obtain more accurate measurements, and thus better quantify the role of ion-neutral reactions in the chemical evolution of molecular clouds.

In H_2 -dominated plasmas, H_3^+ is produced easily by the rapid ion-neutral reaction $\text{H}_2 + \text{H}_2^+ \rightarrow \text{H}_3^+ + \text{H}$. Although previously sought unsuccessfully in interstellar space, the emission spectrum of H_3^+ has been observed in the ionospheres of Jupiter¹², Uranus¹³ and Saturn¹⁴, and possibly also in SN1987A¹⁵. These detections attest to the high efficiency of production of H_3^+ by this reaction and, therefore, to its likely presence in interstellar molecular clouds. In the giant outer planets the ionization of H_2 is performed by accelerated charged particles in the magnetospheres and by solar radiation¹⁶. In the interiors of molecular clouds it is expected to be produced by cosmic-ray particles¹⁻⁴. However, the small concentration of ions (10^{-6} – 10^{-8} of neutrals) and the practical difficulties in observing weak and narrow infrared absorption lines make detection of interstellar H_3^+ difficult.

The present observations of H_3^+ in molecular clouds utilized the deeply embedded young stellar objects GL2136 and W33A, sources of infrared continua, in front of which absorptions in the molecular gas may be studied. These were chosen because the infrared sources are bright and because the large column densities of foreground gas improve the prospects of detecting H_3^+ . In addition, in these clouds some of the gas-phase interstellar molecules which destroy H_3^+ through proton-hop reactions are

known to be depleted by freezing on dust grains^{17,18}, although the depletions are probably small¹⁹.

Measurements of GL2136 and W33A were made using the CGS4 spectrometer at a resolving power of $\sim 15,000$ at the United Kingdom Infrared Telescope (UKIRT) on the nights of 29 April, 10 June and 15 July 1996 (UT). Three vibration-rotation transitions of the ν_2 fundamental band near $3.7 \mu\text{m}$ ($2,700 \text{ cm}^{-1}$) were used for the observations. These start from the lowest H_3^+ *para* level ($J = 1$, $K = 1$) and *ortho* level ($J = 1$, $K = 0$), which lies 22.84 cm^{-1} higher. In April all three absorption lines were detected in the spectrum of GL2136 and were marginally present in the spectrum of W33A, for which the signal-to-noise ratio was lower. In both objects the key *ortho-para* doublet at rest wavelengths 3.6681 and $3.6685 \mu\text{m}$ ($2,726.22$ and $2,725.90 \text{ cm}^{-1}$, respectively) was partially masked by a strong telluric absorption line of CH_4 at $3.6675 \mu\text{m}$ ($2,726.7 \text{ cm}^{-1}$). However, at the times of the subsequent observations the Earth's orbital motion had shifted the doublet further away from the telluric line, making detection easier. Figure 1 shows the spectra of this doublet that were obtained in July, in which the absorption lines are clearly present in both objects. The lines, which are only $\sim 2\%$ deep, have observed widths equal to or slightly greater than the instrumental spectral resolution, indicating that their intrinsic full widths at half maxima are considerably less than 15 km s^{-1} . We derive velocities (with respect to the local standard of rest) for the H_3^+ absorption lines of $25 \pm 5 \text{ km s}^{-1}$ for GL2136 and $29 \pm 5 \text{ km s}^{-1}$ for W33A, in good agreement with velocities of the infrared absorption lines of quiescent cold CO towards these objects, $26.5 \pm 2.8 \text{ km s}^{-1}$ and $32.6 \pm 1.7 \text{ km s}^{-1}$, respectively⁹.

The observed absorptions, derived column densities of H_3^+ in each rotational level, and the total column densities derived from each transition are listed in Table 1. In calculating column densities N we used the standard formula for the equivalent width of an optically thin absorption line

$$W_\nu = \int [\Delta I(\nu)/I(\nu)] d\nu = (8\pi^3\nu/3hc)N|\mu|^2 \quad (1)$$

where $|\mu|^2$ is the square of the transition dipole moment, the values of which were provided to us by J. K. G. Watson.

To calculate the total column density, we assumed thermal equilibrium between *ortho* and *para* H_3^+ at the temperatures of $34 \pm 4 \text{ K}$ and $35 \pm 7 \text{ K}$ for GL2136 and W33A, respectively. These temperatures were obtained from the observed intensity ratios of the close doublet of the $\text{R}(1,1)^+$ and $\text{R}(1,0)$ spectral lines and are significantly higher than the temperatures, 17 K and 23 K , respectively, derived from the infrared spectra of the quiescent CO in those clouds¹⁹. This suggests that the average distances of (gaseous) CO and H_3^+ with respect to the young stellar object may be

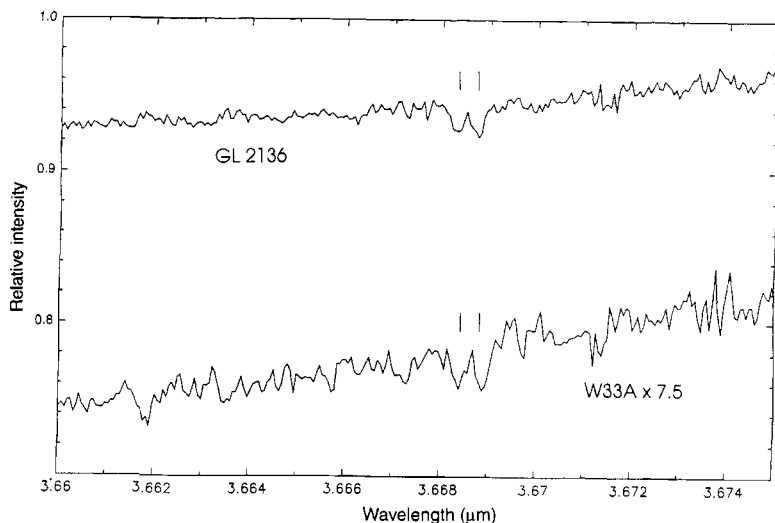


FIG. 1 Spectra of GL2136 and W33A ratioed by BS6378 (A2V), in the vicinity of the H_3^+ *ortho-para* doublet at $3.668 \mu\text{m}$. The doublet, indicated by vertical lines, consists of the $\text{R}(1,0)$ transition of *ortho* H_3^+ at $2,725.898 \text{ cm}^{-1}$ ($3.66852 \mu\text{m}$, right-hand line of doublet) and the $\text{R}(1,1)^+$ transition of *para* H_3^+ at $2,726.219 \text{ cm}^{-1}$ ($3.66808 \mu\text{m}$, left-hand line of doublet).

different, with most of the H_3^+ existing in a somewhat warmer environment than does most of the CO. Higher-quality measurements of these and other lines of H_3^+ are required to confirm this.

The total H_3^+ column densities, $N(\text{H}_3^+)$, are $4.0 \times 10^{14} \text{ cm}^{-2}$ and $6.0 \times 10^{14} \text{ cm}^{-2}$ for GL2136 and W33A, respectively (Table 1). Assuming H_2 column densities of $1.8 \times 10^{23} \text{ cm}^{-2}$ and $2.8 \times 10^{23} \text{ cm}^{-2}$, respectively, determined from the silicate optical depths using the standard gas to dust conversion factor²⁰, we obtain H_3^+ to H_2 concentration ratios of 2.2×10^{-9} and 2.1×10^{-9} for GL2136 and W33A, respectively, which agree approximately with the results of theoretical model calculations²¹ based on an assumed H_2 density of 10^4 cm^{-3} .

These results mark the beginning of direct observations of H_3^+ in molecular clouds. The significance of such measurements is as follows.

First, they provide the most direct evidence supporting the ion-neutral scheme of interstellar chemistry initially proposed by Herbst and Klemperer¹ and by Watson².

Second, the column density of H_3^+ provides a variety of fundamental information, not only about the specific molecular cloud, but also about more universal physical conditions in the interstellar medium. This is because of the approximate equation²² relating the number densities of H_2 , H_3^+ and CO,

$$\zeta[\text{H}_2] \approx k[\text{H}_3^+][\text{CO}] \quad (2)$$

which is obtained by equating the production and destruction rates of H_3^+ . The cosmic-ray ionization flux, ζ , usually is assumed to be $\sim 10^{-17} \text{ s}^{-1}$. The rate constant, k , for the proton-hop reaction from H_3^+ to CO, expected to be the dominant destruction mechanism of interstellar H_3^+ (we neglect slower destruction by the oxygen atoms and other neutrals), has been measured²³ to be $\sim 2 \times 10^{-9} \text{ cm}^3 \text{ s}^{-1}$. Equation (2) is valid in this approximation if the fractional abundance of electrons is less than $\sim 10^{-6}$ and thus dissociative recombination²⁴ of H_3^+ does not compete with proton-hop reactions. As the ratio $[\text{CO}]/[\text{H}_2]$ is approximately constant (at 1.5×10^{-4}) over a wide range of conditions²¹, $[\text{H}_3^+] \sim 3 \times 10^{-5} \text{ cm}^{-3}$ also is approximately constant.

Applying this last result, the concentration ratios of H_3^+ to H_2 derived above for GL2136 and W33A imply that the mean number density of H_2 in each of these clouds is approximately $1.4 \times 10^4 \text{ cm}^{-3}$. The effective length, L , of the cloud in front of the infrared continuum source, derived from $N(\text{H}_3^+)/[\text{H}_3^+]$, is 4 pc for GL2136 and 6 pc for W33A. When equation (2) is written in the form

$$\zeta L = kN(\text{H}_3^+)[\text{CO}]/[\text{H}_2] \quad (3)$$

the observed $N(\text{H}_3^+)$ gives values for ζL of 120 cm s^{-1} and 180 cm s^{-1} , respectively. Independent estimates of L from other observations, such as mapping, can be compared to and combined with the observed values of $N(\text{H}_3^+)$ and $[\text{CO}]/[\text{H}_2]$ to yield a more accurate value for ζ , a parameter which is believed to have the same interstellar value everywhere in the Universe, but for which there are few observational data²⁵. □

TABLE 1 Observed lines and derived column densities of H_3^+

Transition	Frequency* (cm^{-1})	$ \mu ^2$ (D^2)	Source	W_ν (cm^{-1})	$N_{\text{level}}^\dagger$ (10^{14} cm^{-2})	N_{total} (10^{14} cm^{-2})
R(1,1) ⁺	2,726.219	0.0158	GL2136	0.0040 ± 0.0011	2.3 ± 0.5	4.0 ± 0.9
			W33A	0.0063 ± 0.0023	3.5 ± 1.3	6.0 ± 2.2
R(1,0)	2,725.898	0.0259	GL2136	0.0049 ± 0.0011	1.7 ± 0.4	4.0 ± 0.9
			W33A	0.0072 ± 0.0028	2.5 ± 1.0	6.0 ± 2.4
R(1,1) ⁻	2,691.444	0.0140	GL2136	0.0023 ± 0.0014	1.7 ± 1.0	3.0 ± 1.7
			W33A	0.0033 ± 0.0032	2.5 ± 2.4	4.3 ± 4.1

* Laboratory values⁷.

† In lower vibration-rotation level.

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Phase-contrast imaging using polychromatic hard X-rays

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IN conventional radiography, X-rays which pass through an object along different paths are differentially absorbed, and the intensity pattern of the emerging beam records the distribution of absorbing materials within the sample. An alternative approach is phase-contrast radiography, which instead records variations of the phase of the emerging radiation. Such an approach offers improved contrast sensitivity, especially when imaging weakly absorbing samples. Unfortunately, current phase-contrast imaging techniques^{1–11} generally require highly monochromatic plane-wave radiation and sophisticated X-ray optics, so their use is greatly restricted. Here we describe and demonstrate a simplified scheme for phase-contrast imaging based on an X-ray source having high spatial (but essentially no chromatic) coherence. The method is compatible with conventional polychromatic micro-focus X-ray tube sources, is well suited to large areas of irradiation, can operate with a lower absorbed dose than traditional X-ray imaging techniques, and should find broad application in clinical, biological and industrial settings.

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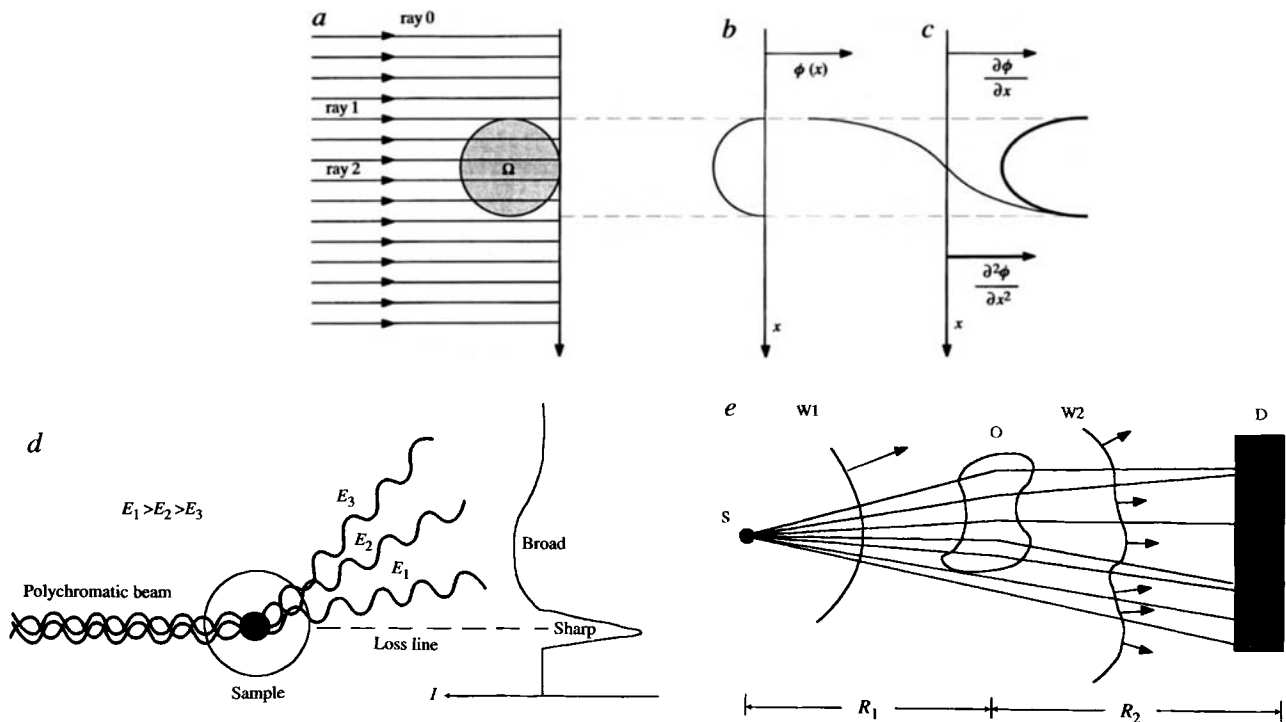


FIG. 1 a–c, Schematic illustration of the formation of edge-contrast for a circular cross-section object, Ω , being irradiated by a parallel X-ray beam. b, The phase difference, ϕ , after passing through the object; c, the phase gradient in the x -direction, $\partial\phi/\partial x$, and the second derivative, $\partial^2\phi/\partial x^2$ (thick curve). The phase difference between ray 2, which passes through the object Ω parallel to the z -axis at constant distance from it, and the reference ray 0 is, from equation (1), $\phi(x, y; k) = -k\delta_\Omega(k)z(x, y)$, where $z(x, y) = 2(r^2 - x^2 - y^2)^{1/2}$ is the length of the intersection of ray 2 with Ω , and r is the radius of Ω . In fact the rays passing through Ω experience refraction at the boundary so that they are not parallel to z . However,

because $\delta_\Omega \approx 10^{-6}$, the above approximate formula for ϕ is quite accurate. d, Schematic illustration of formation of loss-contrast in direction of a ray passing through a boundary feature and showing non-dependence on photon energy. E, such that $E_1 > E_2 > E_3$. e, Schematic illustration of configuration for phase-contrast imaging using a microfocus source (S), an object (O) and a high-resolution two-dimensional detector (D). The spherical wavefront W1 emanating from the point source S becomes distorted to W2 on passing through the object O. R_1 and R_2 are the source-object and object-image distances, respectively.

A key requirement of our method, which resembles in-line holography¹², is high spatial or lateral coherence, which means that the amplitudes of waves are highly correlated between different points transverse to the direction of propagation. The lateral coherence length is $d_\perp = \lambda l / \sigma = \lambda / \gamma$, where l is the source to observation distance, σ is the source size, λ is the wavelength and γ is the angular width of the source as viewed from the observation point. Thus high spatial coherence may be achieved by using a source of small effective size or by observing the beam at a large distance from the source. For 20-keV X-rays, a source size of 20 μm diameter or less and a source-to-image distance of ~ 0.5 m are typically appropriate, giving $d_\perp \approx 1.5 \mu\text{m}$. In general, the smaller the source size the better, provided that the total flux from the source is sufficient.

Variations in thickness and X-ray refractive index of a sample lead to a change in the shape of an X-ray wavefront on passing through the sample. In the geometrical optics approximation, the phase difference, ϕ , for a ray path through an object relative to vacuum is given by

$$\begin{aligned} \phi(x, y; z, k) &= -k \int_{-\infty}^z \delta(x, y, z'; k) dz' \\ &= -\frac{2\pi r_e}{k} \int_{-\infty}^z \rho(x, y, z') dz' \end{aligned} \quad (1)$$

where the optic axis is parallel to z , $k = 2\pi/\lambda$, r_e is the classical electron radius, ρ the electron density of the object, the X-ray refractive index is $n = 1 - \delta - i\beta$, where δ is typically of the order of 10^{-6} for light materials and, say, 10-keV X-rays, so that

refraction angles are usually quite small (a few arc seconds). The phase of the wavefront is taken to be $kz - \phi$ (see ref. 8), so that $\phi < 0$ corresponds to a phase advance. The local propagation vector, $\mathbf{s}(x, y, z)$, is dependent on the gradient of the phase in the direction perpendicular to the local incident wavevector^{7,13} and can be written in the paraxial approximation when $|\nabla\phi| \ll k$ as

$$\mathbf{s}(x, y, z) \approx \left(-\frac{\partial\phi}{\partial x}, -\frac{\partial\phi}{\partial y}, k \right) \quad (2)$$

so that $\mathbf{s}(x, y, z)$ is normal to the wavefront at the point (x, y, z) .

The angular deviation of the normal to the wavefront, $\Delta\alpha$, can be expressed as

$$\Delta\alpha \approx \frac{1}{k} |\nabla_{xy}\phi(x, y, z)| = \left| \nabla_{xy} \int_{-\infty}^z [\delta(x, y, z')] dz' \right| \quad (3)$$

and is thus dependent on the variation of the projected refractive-index perpendicular to the propagation vector, $\mathbf{k}$.

To show that rapid variations in refractive index ('boundary effect') can lead to strong phase-contrast even with polychromatic radiation, we consider the case of a spherical object, Ω , of refractive index $n_\Omega = 1 - \delta_\Omega$ and radius r embedded in a medium of refractive index $n_0 = 1$ (Fig. 1a). The X-ray optical path length differences through the sample lead, via equation (1), to a phase difference $\phi(x, y)$ and hence to a phase gradient $\nabla_{xy}\phi$ in the direction transverse to the direction of propagation (Fig. 1b, c). We then derive from equation (3) the angular deviation of rays through Ω to be

$$\Delta\alpha = \frac{1}{k} |\nabla_{xy}\phi| = 2\delta_\Omega(k) \frac{\sqrt{x^2 + y^2}}{\sqrt{r^2 - x^2 - y^2}}. \quad (4)$$

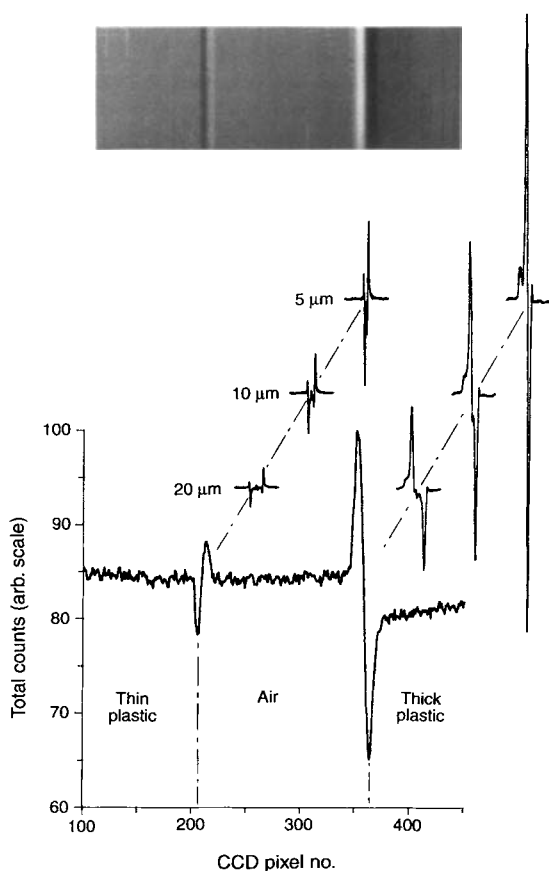


FIG. 2 Top, image of the edge of a thin ($\sim 10\ \mu\text{m}$) and thick ($\sim 100\ \mu\text{m}$) plastic film recorded using a CCD detector (25- μm pixel size) in direct mode and showing sharp contrast variation, $R_1 = 70\ \text{mm}$ and $R_2 = 1,010\ \text{mm}$. The tube voltage was 40 kV. The 10- μm film corresponds to an essentially pure phase object, while the 100- μm film involves a small amount of absorption. Bottom, experimental curve, obtained by integrating the intensity values in the image parallel to the vertical axis, and theoretical curves for regions close to the two plastic-film edges and corresponding to source sizes of 5, 10 and 20 μm . Details of the calculations, which are based on application of the Kirchhoff formula in the spherical-wave case, will be reported elsewhere (A.W.S., manuscript in preparation). Allowance for the polychromaticity of the radiation is made by forming an appropriately weighted sum of monochromatic-case curves. The plastic films were assumed to have a phase difference per unit length of $-55.1\ \text{rad mm}^{-1}$ and a linear absorption coefficient of $0.022\ \text{cm}^{-1}$ for 40-keV X-rays; these values are scaled as λ and λ^3 , respectively, for lower-energy X-rays. The theoretical curves very clearly demonstrate the importance of (small) source size in obtaining phase-contrast information and suggest an actual source size used of between 10 and 20 μm , in good agreement with the nominal source size.

Thus the phase gradient diverges at $x^2 + y^2 = r^2$ (see for example, ray 1 in Fig. 1a), where the rays can deviate by large angles from the optic axis even though δ_n is small. This can lead to an observable redistribution ('loss') in intensity in the corresponding forward direction, the position of which is essentially independent of wavelength (Fig. 1d). More generally, any rapid variations in refractive index or thickness of a sample may be imaged as sharp losses (variations) in intensity at corresponding points in the image even when a polychromatic source is used.

The present experimental configuration for obtaining phase-contrast information in a radiographic image (Fig. 1e) involves a source of high spatial coherence and an X-ray imaging detector, for example film, image plates or a two-dimensional electronic detector. By recording the intensity of the wavefront at a sufficient distance from the sample, intensity variations due to sharp refractive index and thickness variations in the sample may be detected corresponding to a form of differential-phase contrast

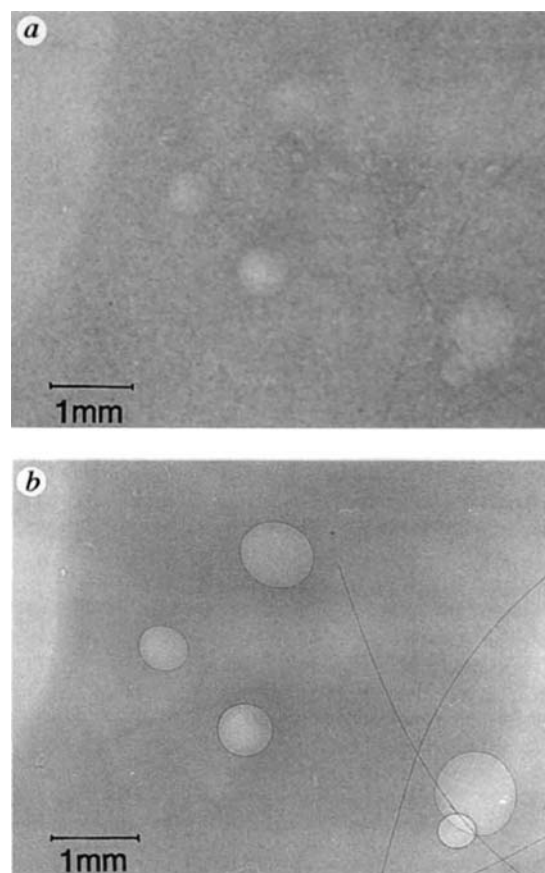


FIG. 3 Images of air bubbles and glass fibres in a polymer glue ('Tarzan's grip', Tarzan's Grip Products, Milperra, NSW, Australia). This is a similar sample to that reported in ref. 8 and corresponds to an almost pure phase object. Source-object distance R_1 was 200 mm, and object-image distances were $R_2 = 1\ \text{mm}$ (panel a; 15-s exposure) and 1,200 mm (panel b; 8-min exposure). The tube voltage used was 60 kV. Image b shows black/white contrast at edges of bubbles and also at edges of fibres, corresponding to additional contrast over that expected for a normal absorption contrast image (a).

imaging. The location of the imaging detector is chosen such that its spatial resolution is sufficient to resolve the intensity differences arising from the wavefront distortions.

A more rigorous treatment of contrast under the present circumstances requires a wave-optical approach based, for example, on the Kirchhoff formula in the Fresnel diffraction case. To a first approximation this leads, for a pure phase object and point source (that is, a spherical wave), to the expression for the intensity of the image^{13,14}

$$I(Mx, My; R_1 + R_2, k) = (I_0/M^2) \left\{ 1 + \frac{R_2}{kM} \nabla_{xy}^2 \phi(x, y; R_1, k) \right\} \quad (5)$$

$$= (I_0/M^2) \left\{ 1 - 2\pi \frac{r_c R_2}{k^2 M} \nabla_{xy}^2 \int \rho(x, y, z) dz \right\}$$

which is valid to first order in $\{(R_2/kM)\nabla^2\phi\}$ assumed small, where $M = (R_1 + R_2)/R_1$ is the magnification of the image (for the usual plane-wave case $M = 1$) and I_0 is the uniform intensity in the object plane. An extended range of validity of equation (5) follows from ref. 15 and A.P., manuscript in preparation.

From the apparently simple formula (equation (5)) two significant conclusions may be drawn: (1) The contrast initially increases with R_2 (that is, increasing defocus from the 'focal conditions' $R_2 = 0$; see also A.P., manuscript in preparation); and (2) the structure of the image is proportional to the laplacian of the projected electron-density and is independent of energy (that is, k) to first order, because for a polychromatic source one

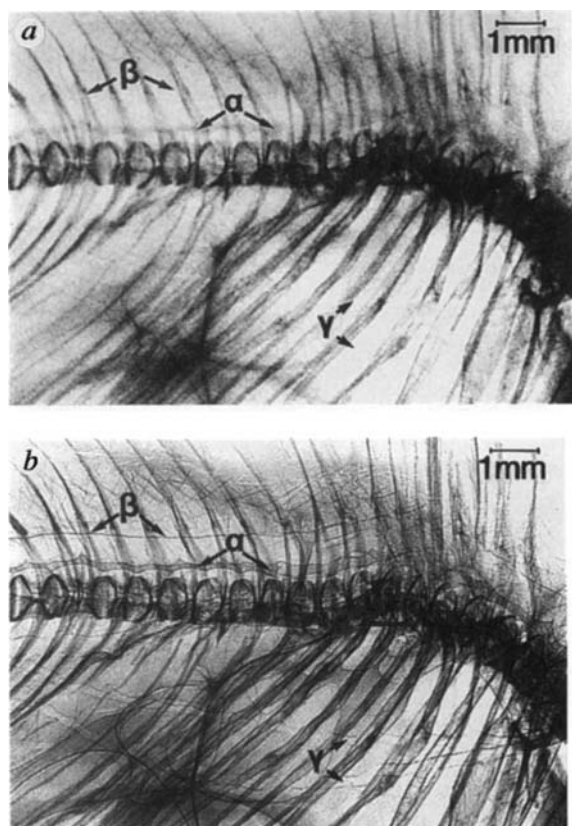


FIG. 4 Image of a small aquarium goldfish (fantail) recorded with a source-object distance of $R_1 = 300$ mm and an object-image distance of $R_2 = 1$ mm (panel a; 2-min exposure) and 1,100 mm (panel b; 110-min exposure). The tube voltage used was 60 kV.

would simply replace $1/k^2$ by a spectrally weighted sum. The quantitative extraction of ϕ (or projected electron density via equation (1)) may be carried out via equation (5) or via solution of a form of transport of intensity equations (refs 11, 16; T. E. G. and S.W.W., manuscript in preparation) or in more general cases from some treatment based on Maxwell's equations, and leads to the possibility of phase-contrast tomography in the present approach.

Examples of images recorded on X-ray film (Agfa Curix) using the present configuration with a source of diameter $\sim 20 \mu\text{m}$ (Kevex model PSX with Cu anode) for various R_2 and R_1 are presented in Figs 2, 3 and 4. We note that for Figs 2 and 3, the samples are essentially pure phase objects and contrast is almost entirely due to the high spatial coherence of the source. A significant feature of the images in Fig. 3 is the presence of sharp dark (loss) lines which resemble normal absorption effects but whose origin is quite different in principle. A conventional fine focus source of diameter 0.1 mm would have a projected size of ~ 0.1 mm relative to the scale bar shown on the photographs and effectively smear out this contrast. Given the additional contrast obtained by increasing R_2 for these simple model objects, it appeared worthwhile to investigate the difference in contrast with increase in R_2 for a biological sample. Thus, Fig. 4 corresponds to radiographs taken of a small aquarium fish (fantail) with source operated at 60 kV, $R_1 = 300$ mm and $R_2 = 1$ or 1,100 mm. The image for $R_2 = 1$ mm corresponds essentially to an absorption-contrast-only image while that for $R_2 = 1,100$ mm should also contain some phase-contrast information. It is very apparent that much more detail of weakly absorbing features of the anatomy of the fish are present in Fig. 4b than the corresponding Fig. 4a. In particular, note the images of the spinal cord (' α '), ligament (' β ') and lateral line canals (' γ '). The fact that contrast at the edges of the organs is negative also points to the presence of phase-contrast in the images. The thickness of the fish was

~ 15 mm. Exposure times used here could have been considerably reduced by the use of more powerful microfocus sources and more efficient detectors, which are available.

The technique described here provides a simple and general method for improving the contrast in radiographs from weakly absorbing features in samples and requires only conventional-type X-ray sources. The method should be particularly relevant to samples with rapid variations in refractive index or thickness. The spatial resolution in the image is essentially determined by the source size. It has the advantage that considerable magnification of the image is possible and so recording media such as image plates may be used, which have many desirable properties such as wide dynamic range and high quantum efficiency but not high spatial resolution. Also, for certain ranges of experimental and sample conditions, the method of image formation is essentially independent of X-ray energy. This means that the sources can be operated at higher tube voltage than normal leading to the ability to penetrate thick samples, and that conditions may be chosen so as, for example, to optimize contrast and minimize dose in clinical applications. □

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Silicon-based visible light-emitting devices integrated into microelectronic circuits

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MICROELECTRONIC device integration has progressed to the point where complete 'systems-on-a-chip' have been realized^{1–3}. Now that optoelectronics is becoming increasingly important for information and communication technologies, there is a need to develop optoelectronic devices that can be integrated with standard microelectronics. Conventional semiconductor technology is largely based on crystalline silicon, which (being an indirect bandgap semiconductor) is an inefficient light-emitting material. This has stimulated significant effort towards developing silicon-

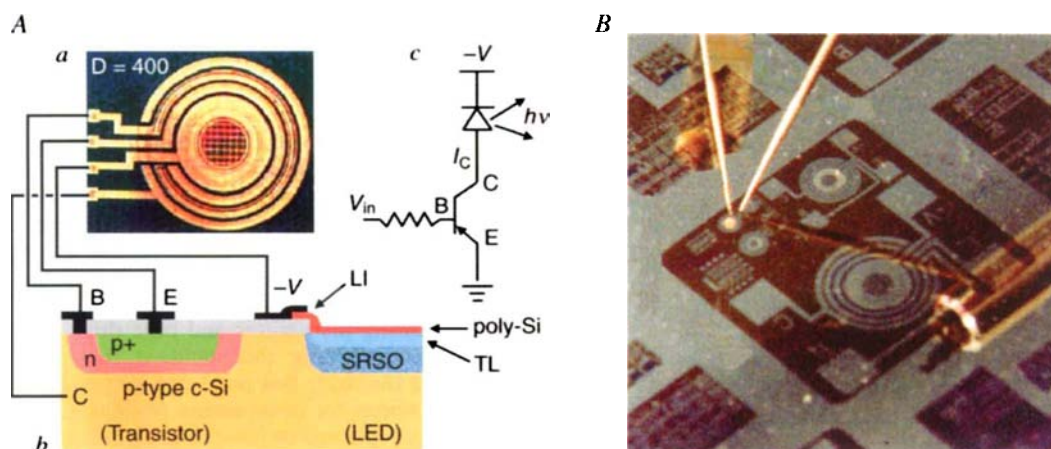


FIG. 1 **A**, Micrograph of an integrated LED/bipolar-transistor structure (**A**, **a**) along with the cross-section (**A**, **b**) and equivalent circuit (**A**, **c**). The LED is in the centre of the structure, and has a 400- μm -diameter active light-emitting area. The micrograph shows (centre) a crosshatched aluminum contact to the LED, which minimizes resistance and promotes uniform current flow through the device. The surrounding bipolar transistor is identified by the concentric emitter (E), base (B) and collector (C) terminals. The cross-section is taken through the centre of the integrated structure and can be mirrored on the right edge because of the symmetric design. The right half of the cross-section shows the multilayer structure of the LED, including the polysilicon (poly-Si) local interconnect (LI) and cathode, the

transition layer (TL) and the active SRSO layer. The left half illustrates the vertical (pnp) bipolar driver transistor, which includes implanted p+ emitter and n-type base regions inside either a bulk p-type crystalline silicon (c-Si) substrate collector (as shown) or a boron implanted p-well collector, which is necessary for electrically isolated structures. **b**, Photograph of a chip under test, showing an integrated LED under forward bias. Only two probes are necessary to bias the LED directly; one contacting the -V terminal, and the other contacting the collector terminal. The mid-sized LED under test has a 320- μm -diameter active area. Light emission is shown to extend over the entire active region. The rest of the chip includes similar integrated structures varying in size from 0.25 to 5 times the device under test.

based optoelectronic components and, of the several strategies explored so far^{4,5}, the use of porous silicon appears the most promising; porous silicon produces high-efficiency, room-temperature, visible photoluminescence⁶, and its material and optical properties have been studied in detail^{7,8}. But the extreme reactivity and fragility of porous silicon have hitherto prevented its integration with conventional silicon processing technology. We have recently shown^{9,10} that the thermal and chemical stability of porous silicon can be greatly enhanced—while retaining desirable light-emitting and charge-transport properties—by partial oxidation. Here we take advantage of these improvements in material properties to demonstrate the successful integration of silicon-based visible light-emitting devices into a standard bipolar microelectronic circuit.

Photodetectors, waveguides, wavelength demultiplexers and modulators have all been fabricated in silicon-based technology¹¹. Although recent progress in hybrid integration with III–V optoelectronic devices has been achieved¹², the technology is expensive and issues of reliability and manufacturability remain⁴. Crystalline silicon p–n junction diodes fabricated by standard microelectronic processing techniques can emit visible light under strong reverse bias, but the reported efficiency ($\sim 10^{-6}\%$) of such devices is extremely low¹³. In this case, light emission is believed to be due to the recombination of electron–hole pairs generated through avalanche breakdown¹⁴, although the mechanism is still under debate¹⁵. The ability to extend the use of silicon as a light-emitting material will enable the realization of all-silicon optoelectronic systems that are compatible with almost all of the microelectronic industry.

We have recently reported^{9,10} on a surface-emitting light-emitting diode (LED) based on silicon-rich silicon oxide (SRSO) which has characteristics among the best reported in silicon-based technology^{16,17} with respect to electroluminescence efficiency, operating threshold conditions and frequency response; this LED demonstrates a significant improvement over porous-silicon-based device structures with respect to device stability. In contrast to porous silicon, the active SRSO layer satisfies several critical microelectronic material processing requirements including tolerance to thermal processing ($\sim 1,000^\circ\text{C}$) and chemical resistance. The fabrication sequence for the SRSO-based LED is

now briefly discussed (a more detailed description of the specific processing conditions is given in ref. 10). A 10 $\Omega\text{ cm}$ p-type crystalline silicon wafer with a heavily doped p+ surface layer is anodized in an HF/ethanol solution. The p+ region is transformed into a mesoporous layer (porosity $\sim 40\%$), whereas the underlying p-type silicon is transformed into a nanoporous silicon layer (porosity $\sim 75\text{--}80\%$) which extends 0.5–1.0 μm into the substrate. An anneal at 800–900 $^\circ\text{C}$ under a dilute oxygen atmosphere (10% O_2 in N_2) is then performed to transform the as-anodized hydrogen passivated porous silicon into SRSO. A 0.3- μm polysilicon film is deposited by low-pressure chemical vapour deposition at 610 $^\circ\text{C}$, and subsequently doped n+ and annealed at 900–1,000 $^\circ\text{C}$ to form the cathode. Aluminum contacts are then made to the n+ polysilicon film and the bulk substrate.

The resulting multilayer structure enables the injection of electrons and holes into the semi-insulating SRSO layer. The thin n+ polysilicon layer provides low contact resistance with minimal light absorption. The density of interface states between the polysilicon and the mesoporous 'transition' layer has been determined to be very low ($< 10^{12}\text{ cm}^{-2}$)¹⁰, thus providing effective carrier transport from the n+ polysilicon cathode to the active light-emitting SRSO layer. Adequate nanocrystallite surface passivation and improved carrier injection have resulted in an increase in the quantum efficiency and significant improvements in the electroluminescence. The SRSO-based devices have the following specifications at room temperature: electroluminescence peak from 1.7 to 2.0 eV; detectable light emission at an applied voltage of $\sim 2\text{ V}$ and a current density of $\sim 10\text{ mA cm}^{-2}$; maximum light intensity of $\sim 1\text{ mW cm}^{-2}$; highest external power efficiency $\sim 0.1\%$; modulation bandwidth exceeding 1 MHz; and stability without degradation for several weeks of continuous operation.

We now report the integration of SRSO-based LEDs into a microelectronic circuit. A simple optoelectronic circuit which integrates the SRSO-based LED with a surrounding bipolar driver transistor has been designed and fabricated (Fig. 1). The driver transistor is connected in a common-emitter configuration and modulates light emission by amplifying a small base input signal and controlling current flow through the LED. Figure 1A shows a micrograph of an integrated structure (Fig. 1A, a) along

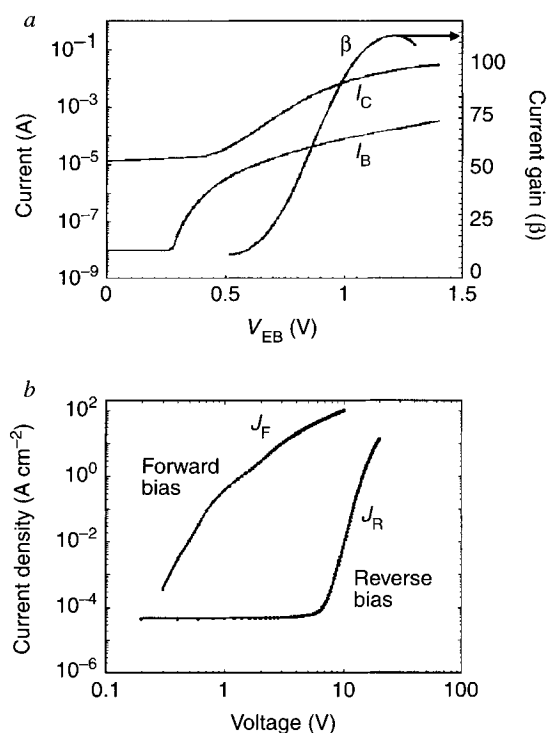


FIG. 2 *a*, pnp transistor Gummel-plot, showing the collector current (I_c) and base current (I_b) as the emitter-base voltage (V_{EB}) sweeps the device into the forward active region of operation. The current gain $\beta = I_c/I_b \approx 100$, which is typical of the standard bipolar process. *b*, LED current-voltage characteristics under forward bias (light-emitting) and reverse bias. J_F and J_R are the forward and reverse bias current densities, respectively. The rectifying ratio (J_F/J_R) is $\sim 10^5$ at 5–10 V bias, the region in which bright electroluminescence is observed.

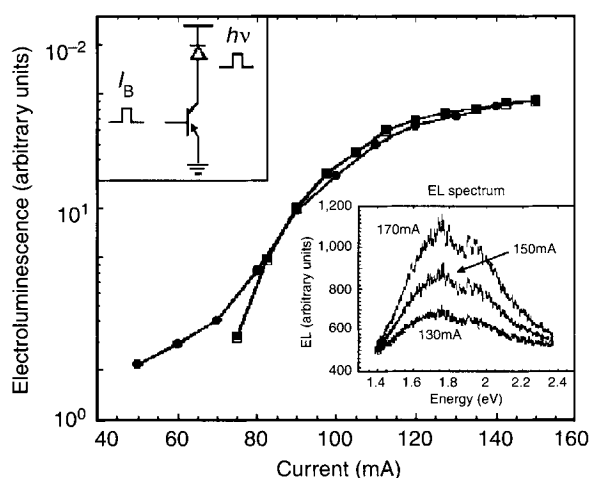


FIG. 3 Electroluminescence response of the LED. The main figure shows the electroluminescence signal as a function of the drive current in both the d.c. mode (circles) and pulsed mode (squares) of operation, with negligible differences. During the pulsed-mode test, a function generator was used to apply a current pulse at 500 Hz with a duty cycle of 10% to the base of the integrated driver transistor. This caused the transistor to drive a current pulse through the LED. The resulting light signal was detected by a photomultiplier tube, which sent a corresponding voltage signal to an oscilloscope that was triggered from the function generator. This ensured that only pulsed light emission which followed the drive current was being measured, as implied by the top-left inset. The bottom-right inset shows that the peak position (near 1.8 eV) and shape of the electroluminescence (EL) spectrum are essentially independent of the applied current.

with its cross-section (Fig. 14, *b*) and equivalent circuit (Fig. 14, *c*). The circular design is area-efficient and scalable, provides effective electrical isolation and demonstrates a truly integrated structure. Various sized structures were fabricated, with the active area ranging from 0.005 to 2 mm².

The standard bipolar process was modified to include five additional lithography levels and several processing steps in order to accommodate bipolar transistor fabrication, isolated SRSO-based LED fabrication, and formation of the transistor-LED local interconnections. The bipolar process used implanted base and emitter regions inside an implanted p-well collector (or a bulk p-type wafer for non-isolated structures). Thermal SiO₂ was grown during the emitter and base drive-in steps for electrical isolation. Following the standard bipolar device fabrication sequence the oxide was patterned and etched, opening windows to bare silicon for construction of the LED. A Si₃N₄ protective layer was deposited by low-pressure chemical vapour deposition, and then patterned to enable formation of selectively anodized regions¹⁸. The LED fabrication sequence, as previously discussed, continued with a high-dose BF₂⁺ implant and anneal (for p+ surface formation), anodization, and SRSO transformation. An n+ polysilicon layer was then produced and patterned to form the LED cathode, and serve as a local interconnection (Fig. 14). Contact windows were patterned and etched through the Si₃N₄ (which remained for electrical isolation) and the underlying SiO₂ layer to the bipolar driver terminals. Last, an aluminum layer was sputtered, patterned and sintered to form the device contacts and interconnect lines. Figure 1*B* shows a photograph of the bright orange electroluminescence emitted by an integrated device on a chip under test.

Electrical and electroluminescence testing of the integrated structure have verified that the performance of both the LED and the bipolar device is consistent with the performance of the individual devices. Figure 2*a* shows the pnp bipolar transistor electrical characteristics. The device exhibits a forward active current gain $\beta \approx 100$, which is typical of the standard bipolar process. The LED current-voltage characteristic (Fig. 2*b*) demonstrates efficient carrier injection under forward bias, with a rectifying ratio of $\sim 10^5$ under light-emitting conditions. Applying a current density of $\sim 100 \text{ A cm}^{-2}$ (corresponding to a total dissipated power of $\sim 1 \text{ kW cm}^{-2}$) for several minutes of continuous operation was nondestructive which indicates effective heat dissipation in the improved device structures. Figure 3 shows the LED electroluminescence response in both d.c. and pulsed modes of operation. The behaviour under d.c. and pulsed operation is

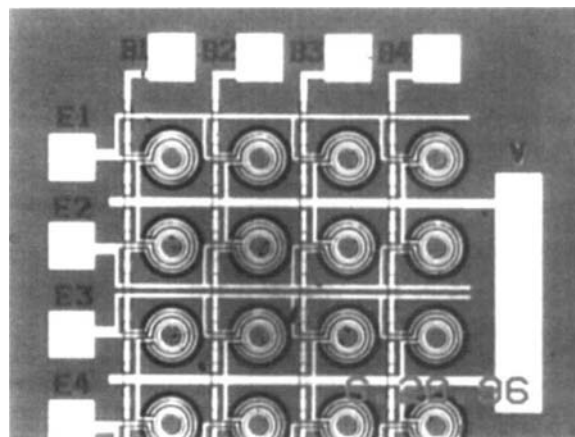


FIG. 4 Micrograph of an addressable LED array using the integrated driver transistors. In the array, each device is the same as the structure shown in Fig. 1, but the LEDs are smaller (active area, 0.005 mm²) and are placed at a pitch of 0.35 mm. An individual LED is addressed by the unique intersection of row (emitter) and column (base) select lines.

almost identical, which confirms that the light is not due to a thermal emission process. Figure 3 bottom-right inset shows the electroluminescence (EL) spectrum at different levels of applied current. This is quite different from the spectra produced from a reverse-biased p-n junction¹⁹ where the electroluminescence spans the entire visible range. The peak position and shape of the spectrum are essentially independent of the applied current. The integrated structures also demonstrated functionality as driver/LED pairs. The drivers were able to amplify low-level base input signals to current pulses of ~100–200 mA, and modulate the LEDs at frequencies in the kilohertz range, which does not represent the upper limit of the LED switching speed as mentioned previously.

Improvements made in the active SRSO light-emitting material and the device design have advanced the LED performance levels towards that of commercial devices, and allowed processing in a standard integrated circuit fabrication sequence without degrada-

tion of either the LED or driver transistor characteristics. There are still significant problems which must be overcome before silicon-based LEDs are recognized as practical light emitters. Improvements in efficiency and power dissipation are necessary for display applications, while increased modulation speed is critical for high speed optical interconnections. In addition, the device structures must be integrated with other optoelectronic components. The development of silicon-based integrated optoelectronic components will enable the incorporation of such structures into advanced systems in order to overcome existing performance limitations. Figure 4 shows an addressable LED array using the integrated local drivers, which could be made much smaller and coupled with peripheral control circuitry. We suggest that the new LED structures could be combined with other silicon-based optoelectronic components and complex microelectronic circuits, thus providing system integration at levels which would otherwise not be possible. □

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Uranium-oxide-based catalysts for the destruction of volatile chloro-organic compounds

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THE industrial release of hydrocarbons and chlorine-containing organic molecules into the environment continues to attract considerable public concern, which in turn has led to governmental attempts to control such emissions. The challenge is to reduce pollution without stifling economic growth¹. Chlorine-containing pollutants are known to be particularly stable, and at present the main industrial process for their destruction involves thermal oxidation at 1,000 °C, an expensive process that can lead to the formation of highly toxic by-products such as dioxins and dibenzofurans². Catalytic combustion at lower temperatures could potentially destroy pollutants more efficiently (in terms of energy requirements) and without forming toxic by-products. Current industrial catalysts are based on precious metals that are deactivated rapidly by organochlorine compounds³. Here we report that catalysts based on uranium oxide efficiently destroy a range of hydrocarbon and chlorine-containing pollutants, and that these catalysts are resistant to deactivation. We show that benzene, toluene, chlorobutane and chlorobenzene can be destroyed at moderate temperatures (<400 °C) and industrially relevant flow rates.

Catalytic combustion of pollutants offers considerable advantages over currently industrially operated thermal combustion. As the catalyst effects oxidative destruction at lower temperatures it is not classed as an incineration process and this eliminates many regulatory requirements. In addition, by virtue of the lower temperature it is more energy efficient and therefore has distinct economic advantages. The major advantage of catalytic combustion, however, is that it can operate with very dilute pollutants (<1%) which cannot be thermally combusted without additional fuel. Hence catalytic oxidation has broader applicability for 'end of pipe' pollution clean-up than does thermal oxidation.

The new catalysts reported here are based on the uranium oxide U₃O₈ which can be used either as a powder or supported on other oxides, either singly or in combination with other oxides that have been found to enhance the performance. In this way a series of uranium-oxide-based catalysts have been designed that are capable of effective destruction of many classes of volatile organic compounds (VOCs). We have selected chlorobenzene as a model chloro-organic compound to demonstrate the effectiveness of the uranium oxide catalysts. Chlorobenzene was selected for three reasons. First, it is listed by the US Environmental Protection Agency as one of the 318 compounds designated as VOCs. Second, chlorobenzene can be considered as a suitable chemical model reagent to probe the destruction of poly-chlorinated biphenyls (PCBs), which are common toxic compounds receiving much media attention⁴. Third, chlorobenzene is a particularly stable molecule that is difficult to oxidize owing to its stabilization by aromaticity. Few previous studies have tried to oxidize such a stable molecule and most have concentrated on the study of the oxidation of non-aromatic chloro-organic compounds, such as methylene chloride⁵. We also present results for chlorobutane destruction over a uranium oxide catalyst, indicating that these catalysts have wider applications for destruction of chlorinated VOCs.

The oxidation of chlorobenzene was carried out under condi-

tions appropriate for the treatment of industrial effluent streams. Chlorobenzene (1% by volume in air) was passed over the oxide catalyst (~0.2 g) using a conventional fixed-bed plug-flow micro-reactor so that the total gas flow was 70,000 ml STP gas per h per ml catalyst. Product analysis was carried out using standard gas chromatography and mass spectroscopic techniques. The U_3O_8 catalyst was prepared by thermal decomposition of the $UO_2(NO_3)_2 \cdot 6H_2O$ salt, whilst supported catalysts were prepared by impregnation of fumed SiO_2 by uranyl nitrate solution. The active loadings were 10 mol% and the precursor was dried at 100 °C for 24 h before calcination at 300 °C for 1 h and then at 800 °C for 3 h. The catalytic performances of the uranium oxide catalysts, in non-supported and supported form, are shown in Table 1. At 350 °C the pure uranium oxide, U_3O_8 , is found to be most effective and the inlet chlorobenzene concentration (10,000 parts per million, p.p.m.) is reduced to only 31 p.p.m.; this represents a conversion of 99.7%, which is comparable to the currently used thermal oxidation processes operating at >1,000 °C. It is important to note that the only products observed from the conversion of chlorobenzene over the uranium oxide catalysts are carbon oxides and HCl; the latter can be readily removed by treatment with water of the gases leaving the catalytic converter. No other products were detected even in trace amounts and hence this catalytic conversion offers considerable advances over other catalytic and thermal oxidation processes, which may also give trace levels of toxic by-products (for example, partially oxidized chloro-organics, dioxins and benzofurans). It is interesting to note that at 350 °C the oxide UO_3 is inactive, as is the oxide Co_3O_4 which has been found to be highly effective in previous studies for the oxidation of VOCs that are not chloro-organics⁵. Co_3O_4 only becomes active for this reaction at temperatures >400 °C. Supporting U_3O_8 on silica is found to slightly decrease the effectiveness; this is probably due to the dilution of the active uranium oxide surface by the silica support. The addition of Co to the U_3O_8/SiO_2 catalyst restores the activity at 400 °C (exit chlorobenzene concentration, 11 p.p.m.) to the level of U_3O_8 , but other oxides are not such effective additives. The catalytic performance of the uranium oxide catalyst is not diminished by continued use, showing no evidence for deactivation after 400 h of use. This is in marked contrast to the precious-metal-based catalysts that are currently commercially available for the oxidative destruction of VOCs (for example, Pt/SiO_2); these are known to be rapidly deactivated on exposure to chlorine-containing molecules⁶. In this context it is important to compare the catalytic performance of the uranium-based catalysts with previously reported catalysts for the oxidative destruction of VOCs, and these comparisons are given in Table 2. The only previously reported attempt to oxidize chlorobenzene⁷ achieved only 92% conversion even when a much higher temperature of 530 °C was used with a Pt/SiO_2 catalyst. The best catalyst performance reported² to date for the destruction of chloro-organics was obtained using $CuCl/KCl/SiO_2$ catalysts. These catalysts operate at similar temperatures to our uranium-based catalysts but require the use of very low flow rates (300 ml

TABLE 1 Chlorobenzene destruction over powdered catalysts

Catalyst	Temperature (°C)	Conversion (%)	CO _x selectivity (%)
U_3O_8	350	99.7	100
U/SiO_2	400	99.9	100
$Co/U/SiO_2$	400	99.9	100
$Cr/U/SiO_2$	400	99.9	100
$Cu/U/SiO_2$	400	99.9	100
$Fe/U/SiO_2$	400	99.9	100
$Ni/U/SiO_2$	400	89	100
$Mn/U/SiO_2$	450	99.9	100
Co_3O_4	600	62	100

Experiments performed at a total gas flow of 70,000 ml STP gas per h per ml catalyst.

* Defined as (carbon oxide yield/total product yield) × 100.

gas per h per ml catalyst). Comparison with our new uranium catalysts indicates that over 230 times more VOC can be destroyed than with the Cu-based catalyst in any fixed time period. It is clear from these comparative data that the uranium-oxide-based catalysts offer a significant advance in the design of a new process for the catalytic destruction of VOCs.

The uranium oxide catalysts are also equally effective for the oxidation of other classes of VOC. Representative data are given in Table 3 for the oxidative destruction of benzene, toluene, butane, cyclohexanone and butylacetate. In the case of benzene, a particularly harmful environmental pollutant and a known carcinogen, total conversion was achieved using the U_3O_8/SiO_2 catalyst at 400 °C; further addition of Cu to this catalyst maintained the oxidation activity of the supported catalyst. We have found that by using the supported form of uranium oxide we can tailor catalyst formulations for a particular effluent treatment and each variant is capable of long-lived high conversion for that particular duty. For example, the total oxidative destruction of cyclohexanone could be achieved at temperatures as low as 300 °C with U_3O_8 catalyst, and butylacetate could be totally oxidized at 350 °C with the $Cu/U_3O_8/SiO_2$ catalyst. These are temperatures at which other known oxidation catalysts, for example, Co_3O_4 , are inactive.

Here we have described the use of U_3O_8 and U_3O_8/SiO_2 catalysts and given results from the testing of these catalysts in a conventional microreactor. In an industrial application, the reactor will have to handle high gas velocities with very little pressure drop. Schematic reactor designs for catalytic VOC destruction have been proposed¹ in which the catalysts may be either supported on a monolith or be present in beaded form. In the case of a monolithic reactor the catalyst can be added as a wash coat to the monolith support; this often leads to relatively low active surface areas but prevents catalyst carryover in the exhaust stream. The use of a catalyst in beaded form often exposes higher active

TABLE 2 Comparison of catalytic performance for the destruction of chloro-organic compounds

Catalyst	Chlorocompound	Concentration (p.p.m.)	Temperature (°C)	Gas flow (h ⁻¹)	Conversion (%)	Exit concn (p.p.m.)	Ref.
U_3O_8	Chlorobenzene	10,000	350	70,000	99.7	31	This work
U_3O_8	Chlorobutane	10,000	350	70,000	>99.5	<50	This work
$CuCl/KCl/SiO_2$	Methylene chloride	10,000	350	300	98.4	160	2
0.1%Pt/ Al_2O_3	Chlorobenzene	398	530	30,000	92.0	32	7
Co-Y	Trichloroethane	1,500	325	2,361	100	—	9
Cr_2O_3	Trichloroethane	10,000	650	3,600	99.7	30	10
TiO_2	CF_2Cl_2	2,000	400	10,500	98.0	40	11
WO_3/Al_2O_3	CF_3CF_2Cl	6,700	600	15,366	60.0	2,680	12

* Defined as ml STP gas per h per ml catalyst.

surface areas, but catalyst erosion and carryover are more common. For these reasons it is envisaged that the catalyst detailed here would be used in supported form.

Uranium-based catalysts have been used industrially for many years⁸. There are well established procedures for their safe handling which are determined primarily by chemical toxicity considerations. Suppliers also recognise that these materials will not be used unless the burden of compliance with other relevant regulations is kept to a minimum. One way of achieving this is to return spent catalysts for suitable retreatment via well established chemical processes. Our results provide an effective demonstration that uranium oxide, considered by many as an environmental burden, can be used to provide an answer to one of the environmental problems of today. The industrial commercialization of these catalysts is now being actively pursued. □

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TABLE 3 VOC destruction over uranium catalysts

Catalyst	VOC type	Temperature (°C)	Conversion (%)	CO _x selectivity (%)
U ₃ O ₈ *	Benzene	400	99.9	100
U/SiO ₂ *	Benzene	400	99.9	100
Cu/U/SiO ₂ *	Benzene	400	99.9	100
U/SiO ₂ †	Toluene	400	99.9	100
U ₃ O ₈ *	Butane	600	81	100
U/SiO ₂ *	Butane	500	99.9	100
Cu/U/SiO ₂ *	Butane	450	95	100
U ₃ O ₈ †	Cyclohexanone	300	99.9	100
U/SiO ₂ †	Cyclohexanone	300	99.9	100
U ₃ O ₈ †	Butylacetate	350	99.9	100
U/SiO ₂ †	Butylacetate	350	99.9	100
Cu/U/SiO ₂ †	Butylacetate	350	99.9	100

Experiments performed at a gas flow rate of 70,000 ml STP gas per h per ml catalyst. VOC, volatile organic compound.

* Pelleted catalyst.

† Powdered catalyst.

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Highly concentrated seismicity caused by deformation of Kilauea's deep magma system

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FREQUENT shallow earthquakes within the rift zones of the Hawaiian volcano Kilauea have been interpreted as resulting from stress changes associated with a shallow magma conduit system^{1,2}. Here, by using a precise earthquake relocation technique³, we show that what had been imaged as a diffuse cloud of seismicity in the Upper East Rift in 1991 is in fact a narrow ribbon, defining vertical strike-slip faults that extend 2.5 km along the rift, but less than 100 to 200 m vertically. This extreme aspect ratio in the seismicity is unexpected and has not been recognized in other fault systems. The observed earthquake depths, left-lateral focal mechanisms, limited vertical extent and increasing moment release rate since 1983 can be explained in terms of a growing stress concentration above the deeper, aseismic portion of Kilauea's rift system, which deforms in response to the seaward displacement of the south flank of the volcano. The shallow background seismicity within Kilauea's rifts can therefore be tied to large-scale motions of the volcanic edifice, and need not be interpreted as resulting from magma migration. The observed pattern of seismicity may also have implications for the mechanical erosion of locked patches along major strike-slip faults elsewhere.

Kilauea's rift zones are believed to be sites of persistent magma storage bodies as well as repeated shallow dyke intrusions and fissure eruptions. Geodetic studies^{4,5} have located potential magma bodies at (poorly constrained) depths of 3–5 km, and petrological studies⁶ have identified eruptions that tapped batches of magma that differentiated within the rift. In many cases rift-zone eruptions precede subsidence of the summit reservoir by

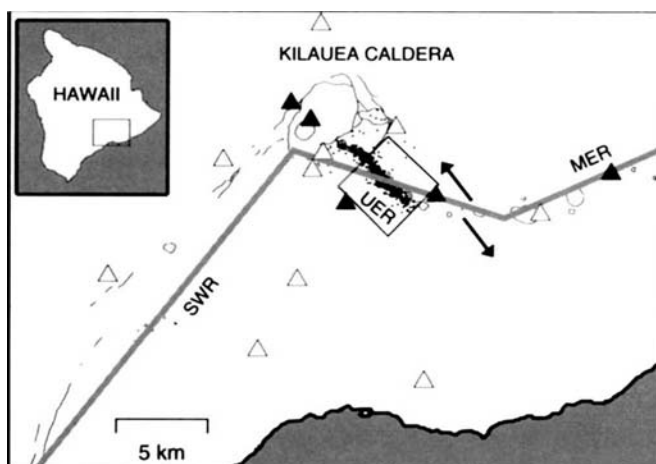


FIG. 1 Map of Kilauea showing catalogue locations of the 826 earthquakes (dots) examined in this study and the box containing the three largest multiplets found. Triangles show commonly used seismic stations; those filled are three-component stations. Stippled line segments show the dislocations found by Delaney et al.¹⁷ to model deep motion within the Southwest, Upper East, and Middle East rifts (SWR, UER and MER). The strike of the relocated seismicity is rotated ~20° clockwise from the UER dislocation, but the geodetic data probably cannot resolve this difference¹². Arrows show inferred sense of relative motion across the deep UER¹⁷.

several hours, suggestive of a magma pathway along the rift⁷⁻⁹. Although not well understood, shallow rift-zone earthquakes have generally been interpreted as reflecting host-rock deformation caused by dyke intrusion or magma pressure changes within this shallow conduit system. A close association between magma transport and seismicity is clear during shallow dyke propagation, when geodetic data place the dyke at about the same depth as migrating earthquake swarms (~2–4 km depth^{2,10}). The relationship is less clear for background or swarm-like seismicity occurring in the absence of known dyke intrusions. Also unclear is the relationship of the shallow rift zone to the aseismic region beneath it, which apparently undergoes displacements of 20 cm per year or more^{11,12}. This deforming zone is believed to extend down to the old sea floor, essentially decoupling the south flank of Kilauea from the region to the north. We refer to this deep region—which may consist in part of partially molten rock—as the deep rift system^{13,14}.

To better characterize shallow seismicity within Kilauea's rift zones, we relocated earthquakes from among 826 that occurred in 1991 within the Upper East Rift (UER; Fig. 1). With the exception of a (non-migrating) 69-event swarm in August, the seismicity represents fairly steady activity. To relocate the earthquakes we used the algorithm of Got *et al.*³, which consists of identifying multiplets (families) of similar earthquakes by waveform cross-correlation, and then using cross-correlation to obtain precise relative arrival times for each pair of events within the multiplet. The relative P- and S-wave arrival times at all stations are then combined in a linear inversion for event relative location and origin time. Physically, for two earthquakes to appear similar it is necessary that they have similar focal mechanisms and locations.

Figure 2a, b shows the Hawaiian Volcano Observatory (HVO) catalogue locations of 345 earthquakes contained in three multiplets; all were located within the box straddling the UER in Fig. 1, which contains 657 events (see Methods for a discussion of the multiplet selection process). The seismicity forms a 'cloud' nearly 1 km wide and 1–2 km high. After relocation, the three multiplets

define linear trends only 10–100 m wide (Fig. 2c). The composite fault-plane solutions of the two largest multiplets are consistent with double-couple focal mechanisms, and in conjunction with the strikes of the relocated events show them to be left-lateral strike-slip faults. In cross-section, each multiplet barely exceeds 100 m in height, and from the average of the catalogue locations all three are located at close to the same depth (Fig. 2d). The median magnitude of the events (1.3) is such that the expected rupture diameter is roughly 100 m (refs 15, 16); for events of this size we are relocating the earthquake centroids. The limited vertical extent is not an artefact of the relocation method because re-picking the aligned traces visually and using standard location techniques yielded a similar, albeit less sharp, image. The multiplets contain more than 50% of the seismicity in the box in Fig. 1, and in the region of the southeastern multiplet more than 70% of the catalogue has been relocated. In addition, the catalogue depth distribution of the selected events is similar to that for events not selected in any multiplet. This suggests that we are not systematically excluding large numbers of significantly shallower or deeper earthquakes from the relocation.

We propose that this narrow band of seismicity is generated by left-lateral motion of the underlying deep rift system as the latter deforms to accommodate the southeasterly displacement of Kilauea's south flank. If, as suggested by the extremely rapid deformation rates, the rift system at depths greater than several kilometres is hot and weak, one expects a stress concentration along the colder, shallow UER where rocks are brittle and relatively strong. Accordingly, we examine the earthquakes from the point of view of the stress concentration at the tip of a deforming crack in an elastic body, a rough approximation of the form and role of the deep rift system. We envision that the relocated seismicity occurs along an existing fault overlying the deep rift or the combined deep-rift/shallow-conduit complex. The left-lateral focal mechanisms agree with the sense of motion across the deep rift system inferred from geodetic data, and the depth of the relocated seismicity (3.1 km) is compatible with the

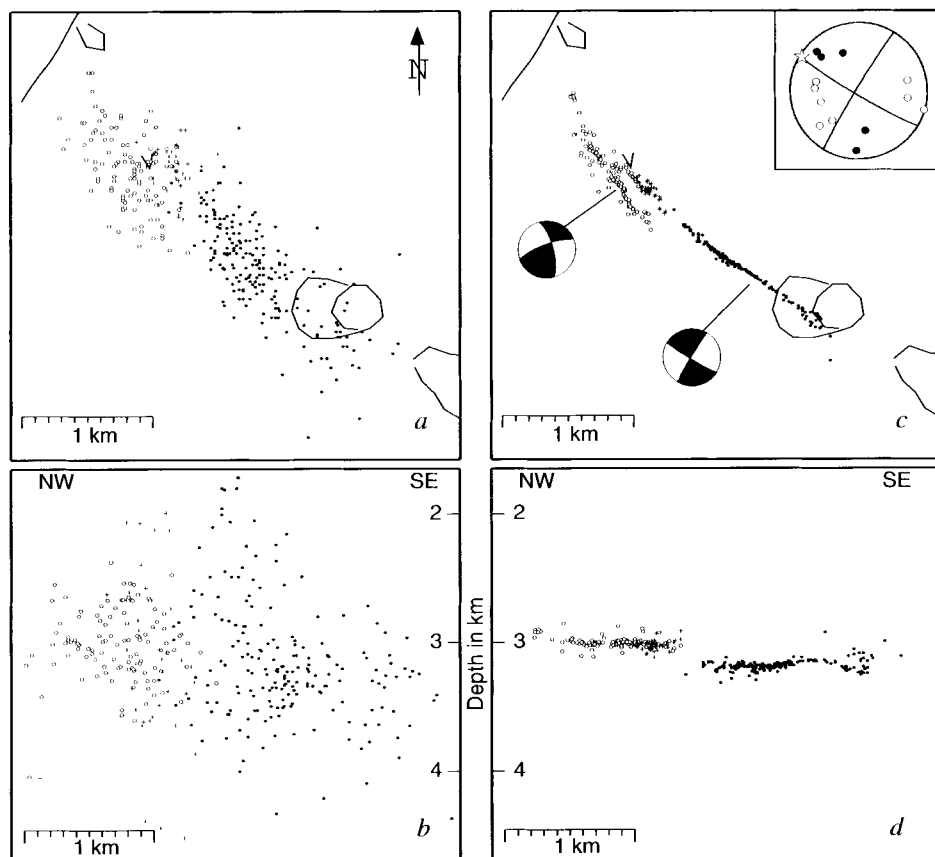


FIG. 2 Seismicity before and after relocation. a, Map view of three multiplets (filled circles, open circles, and crosses) extracted from the 1991 HVO internal catalogue. b, Cross-sectional view of the seismicity looking northeast. c, Map view of the three multiplets following relocation. The beach balls represent well-constrained composite focal mechanisms with the compressional quadrants in black. Inset, first motion polarity for the southeastern multiplet. A single station (star) has emergent as well as clear compressional and dilatant arrivals and constrains one of the nodal planes. d, Cross-sectional view following relocation. The relative locations of the two adjacent multiplets to the northwest are constrained by four shared events. The absolute locations of the two largest multiplets are determined by the average of the catalogue locations.

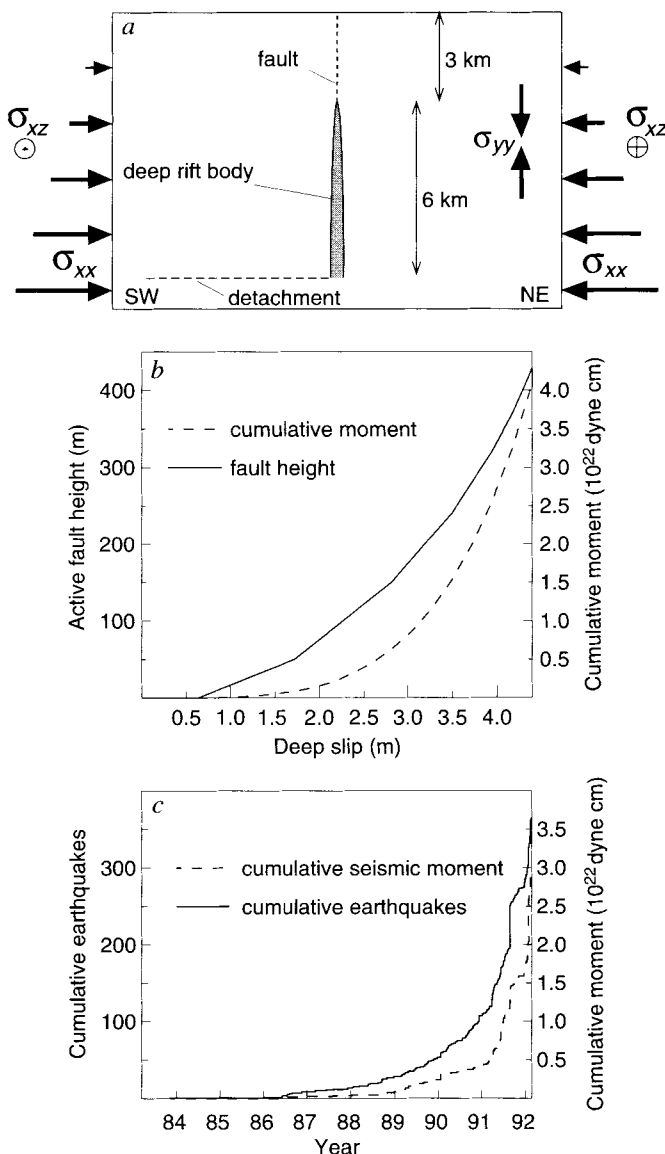


FIG. 3 a, Schematic diagram of boundary-element model. The shallow fault is modelled by a line of elements obeying Coulomb friction. The effect of the deep detachment is approximated by doubling the height of the deep rift body so that its bottom lies 6 km below the detachment. b, Calculated active shallow fault height (solid curve) and total moment release (dashed curve) plotted as a function of strike-slip displacement of the deep rift body, assuming that initially $\sigma_{xz} = 0$ and $\sigma_{xx} = \sigma_{yy}$ along the shallow fault. An along-strike length of 2,500 m is used for the moment calculation. We assume an elastic shear modulus G of 10 GPa; if the actual value is X times that, the coordinate values along the horizontal axis should be divided by X . Approximately 2 m of left-lateral slip occurred from the end of 1982 (when we infer that the above stress state was imposed on the shallow fault) to 1991. c, Cumulative number (solid curve) and moment release (dashed curve) of earthquakes located within the box in Fig. 1, contained in the HVO published catalogue from January 1983 to February 1992. The catalogue completeness did not change during this time. The internal catalogue, which comprised the database for the relocation and contains many smaller events, shows the same trend but extends back only to 1986. The published catalogue contains the larger events and most of the moment release. Seismic moment is estimated from the moment-magnitude relation of Savage and Meyer¹⁵ for earthquakes beneath the south flank of Kilauea.

the deep rift as a weak crack extending from 3 to 9 km depth and imposes the small increase in far-field stress necessary to drive this motion, the frictional strength of an overlying vertical fault is exceeded over a vertical extent of the order of 100 m. An approximate analytic expression for this vertical extent, R , is $R = (w^2 G^2) / (2h \Delta\sigma_\tau^2)$, where w is the geodetically determined (average) deep strike-slip motion that drives the shallow seismicity, G is the elastic shear modulus, h is the height of the deep rift, and $\Delta\sigma_\tau$ is the increase in shear stress required for frictional slip along the shallow fault¹⁸. Frictional slip requires that $\sigma_\tau = \mu(\sigma_n - p_f)$, where σ_τ is the shear stress, μ is the coefficient of friction, σ_n is the normal stress and p_f is the pore fluid pressure¹⁹. At 3 km depth, with σ_n equal to the vertical stress (~ 75 MPa), σ_τ initially zero, p_f hydrostatic (30 MPa) and $\mu = 0.6$, we find $\Delta\sigma_\tau = 27$ MPa. Assuming²⁰ $G = 10$ GPa, this yields $R = 50$ m for 2.25 m of left-lateral displacement at depth. A numerical boundary-element calculation, that includes both the influence of the free surface and the unloading of the fault due to the lesser opening displacement of the deep rift (7 cm yr^{-1}), increases this estimate to 100 m (Fig. 3a, b). Although the precise numbers depend upon the choice of the elastic stiffness and initial stress, the plausibility of this mechanism is clear. Because the height of seismicity is much less than the height of the deep rift 'crack', the results depend essentially on the magnitude of deep motion and not how that motion is driven (that is, by stress changes within the deep rift system or in the far field).

In addition to providing an explanation for the limited vertical extent of seismicity in 1991, this model predicts that the active fault height and moment release rates should increase with time—approximately as second and fourth powers of the deep displacement, respectively (Fig. 3b). Although we do not have the resolution to see an increase in fault height in 1991 (in fact the expected increase is only ~ 20 m), these predictions can nonetheless be tested independently using the published earthquake catalogue. Figure 3c shows the cumulative number of earthquakes and the cumulative seismic moment from the study area, following the aftershocks of the December 1982 intrusion and before another small intrusion in March 1992 that apparently altered the stress again. Despite the nearly constant deformation rate until the end of 1990¹⁷, the earthquake production and moment-release rates increase steadily with time, with only one event recorded before 1986. The magnitude (M) of the largest earthquakes also increases with time, with the first (of 29) $2.5 < M < 3$ events occurring in 1989, and the first (of 7) $3 < M < 3.5$ events occurring in 1991. As earthquake magnitude is strongly dependent on the minimum fault dimension¹⁶, this is also consistent with the active portion of the fault increasing in height. Last, the estimated seismic moment release of 0.5×10^{22} dyne cm from 1983 until early 1991 matches that calculated from the boundary-element

inferred depth of the top of the deep rift (3 km)¹⁷. The limited vertical extent of seismicity is also consistent with failure within the stress perturbation of a deforming crack, provided that the background stress away from the crack tip is not near failure. Dyke intrusion is an appealing mechanism for generating a background stress state far from failure, because at points near to the dyke and well below the dyke top any shear stress along rift-parallel planes is relaxed to essentially zero, and the normal stress is increased to the level of the magma pressure. Twice in 1980 and again in December 1982 this portion of the UER generated migrating earthquake swarms, simultaneous with subsidence of the summit region, that have been interpreted as indicating dyke intrusion²; we adopt that view here. A significant fraction of these earthquakes have catalogue depths that are considerably shallower than those from 1991, consistent with the tops of the dykes being emplaced near the surface.

Geodetic data from 1983 to 1991 analysed by Delaney *et al.*¹⁷ indicate that the rate of deformation of Kilauea was quite steady from the end of 1983 until the end of 1990. They modelled the deep rift system beneath the UER as undergoing 26 cm per year of predominantly left-lateral strike-slip displacement during this period (Fig. 1). Projecting this displacement-rate vector onto the strike of the relocated multiplet and extrapolating to the end of 1991 yields a total left-lateral slip of 2.25 m. If one treats

model when the active fault height reaches 140 m, which would occur at 2 m of deep strike-slip motion if G were 13 GPa rather than 10 GPa (Fig. 3b).

Because such a high proportion of the total earthquake population was selected as belonging to multiplets, it seems most plausible that we are imaging the boundary of a locked fault being eroded by creep at greater depth, rather than formation of a new fault. The extreme concentration of seismicity begs the question of whether similar behaviour might be observed surrounding locked patches of major strike-slip faults elsewhere. An essential ingredient of the proposed model is that the initial tractions on the fault be relatively uniform as well as far from failure. The most plausible time for encountering such stress states on faults outside volcanic regions would be following major earthquakes. Whether the residual stresses following such events could be sensibly uniform is currently a topic of considerable interest^{21–24}. Thus searching for similar patterns of seismicity along major strike-slip faults could shed light on the tectonic earthquake cycle. Within Kilauea's rifts the 'earthquake cycle' is likely to be reset by dyke intrusion before fault slip reaches the surface. □

Methods

The measure of similarity of two traces is the average coherency, in the frequency range 4–13 Hz, of a 1.28 s (128 time samples) window containing the P wavetrain. Two events are considered similar if this average coherency averages more than 90% at four nearby stations. A multiplet is defined as a family of earthquakes in which each event is similar to at least n others. The multiplets described in this study are the three satisfying the requirement that each event is similar to at least 15 others. Although each event need not be similar to all other events in the multiplet, at some stations (in general those lying within the middle of the quadrants of the focal sphere) the traces are coherent across an entire multiplet. Relaxing the multiplet standards increases the size of the multiplets but decreases (on average) the accuracy with which events can be relocated; in our case decreasing n to 10 generates a single multiplet containing 425 events that spans the entire box straddling the UER in Fig. 1. Using the method of VanDecar and Crosson²⁵, which estimates cross-correlation errors from the internal consistency of all the redundant delay measurements, the average accuracy of the time delays for the three multiplets ranges from 0.8 to 1.7 ms (0.08 to 0.17 samples) at the nine best stations. This is 25–50 times the accuracy with which individual P-wave arrival times can be measured. For a P-wave velocity in the source region of 5 km s⁻¹ (ref. 26) this is consistent with a relative location accuracy of 5–10 m in horizontal position and 10–20 m in depth. The r.m.s. differences between the measured time delays and those computed from the three relocations range from 3.9 to 4.4 ms. Most of this misfit is likely to come from errors in the assumed take-off angles and violation of the assumption that the azimuth and take-of angle from every member of the multiplet to a given station is identical³.

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High intrinsic rate of DNA loss in *Drosophila*

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PSEUDOGENES are common in mammals but virtually absent in *Drosophila*¹. All putative *Drosophila* pseudogenes show patterns of molecular evolution that are inconsistent with the lack of functional constraints^{2–5}. The absence of bona fide pseudogenes is not only puzzling, it also hampers attempts to estimate rates and patterns of neutral DNA change. The estimation problem is especially acute in the case of deletions and insertions, which are likely to have large effects when they occur in functional genes and are therefore subject to strong purifying selection. We propose a solution to this problem by taking advantage of the propensity of retrotransposable elements without long terminal repeats (non-LTR) to create non-functional, 'dead-on-arrival' copies of themselves as a common by-product of their transpositional cycle^{6–8}. Phylogenetic analysis of a non-LTR element, *Helena*, demonstrates that copies lose DNA at an unusually high rate, suggesting that lack of pseudogenes in *Drosophila* is the product of rampant deletion of DNA in unconstrained regions. This finding has important implications for the study of genome evolution in general and the 'C-value paradox' in particular.

Non-LTR retrotransposable elements comprise a distinct group in the retroid lineage¹⁰. These elements are on average 4–5 kilobases (kb) long, have no introns, lack terminal repeats, have a dA-rich tail at the 3' end, and contain 1–2 open reading frames, one of which encodes the reverse transcriptase. Transposition of these elements often results in the creation of 5'-truncated copies^{6–8}. Such copies lack a promoter and do not encode functional proteins. These 'dead-on-arrival' copies are expected to manifest patterns of neutral evolution because they are not subject to selection for the ability to transpose; they are effectively pseudogenes.

We have studied the evolution of a particular non-LTR retrotransposable element, *Helena* (Fig. 1), which was originally cloned as one of several transposable elements activated in hybrid dysgenesis in *D. virilis*¹¹. We have sequenced 18 different copies of *Helena* from 8 species in the *D. virilis* group. The sequenced region comprises 363 base pairs (bp) at the 3' end of the putative reverse transcriptase. Alignment of these sequences revealed a low amount of sequence divergence and an unexpectedly large number of deletions (Fig. 2a). We have observed no internal insertions relative to the consensus. All deletions are unique; in fact, no two deletions share the same breakpoint. Figure 2b shows the distribution of the deletion sizes: deletions range from 1 to 75 bp and average 24.3 ± 23.1 bp.

To gain insight into the evolutionary history of the sampled copies of *Helena*, we have performed phylogenetic analysis using maximum parsimony¹² with the nucleotide sequence alignment.

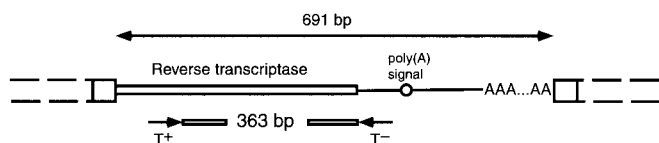


FIG. 1 Sequence organization of the insertion of *Helena* in the *singed* locus in *D. virilis*. It is 691 bp long and truncated at the 5' end. It contains an open reading frame encoding a part of the putative reverse transcriptase, followed by a poly(A)-addition signal and a 41-bp stretch of poly(A). T⁺ and T⁻ are the primers used to obtain sequence from multiple copies of *Helena*.

Figure 3a shows the resulting tree. The tree can be used to classify all substitutions into shared substitutions, which map to the internal branches of the tree, and mostly unique substitutions, which map to the terminal branches. The internal-branch substitutions can be interpreted as having occurred in the active lineages of *Helena*. Accordingly, the terminal-branch substitutions would be those that arose either in the active lineage that gave rise to a single sampled copy, or in the sampled copy itself since its insertion. When the number of active lineages is small and the sampling is relatively dense, the majority of terminal-branch substitutions will have occurred after transposition. This pattern is expected because most of the active lineages will have given rise to multiple sampled sequences, and the substitutions in active lineages will appear as shared among members of the sample and will map to the internal branches.

These assumptions can be tested by examining the patterns of purifying selection in the distribution of terminal-branch and internal-branch substitutions. The internal-branch substitutions, if they truly correspond to the substitutions in the active lineages, should show evidence of purifying selection at the amino-acid level, because the sequenced region corresponds to a functionally important and constrained protein, reverse transcriptase^{13,14}. On the other hand, the terminal-branch substitutions should show a more or less random pattern of substitutions. As most of the substitutions in the third position of codons are synonymous, and the substitutions in the first and second position are usually non-

synonymous, we expect that most of the internal-branch substitutions will map to the third positions and that the terminal-branch substitutions will map randomly to all three positions. This is exactly what we observed (Fig. 3b): there is an excess of third-position substitutions along internal branches (χ^2 test, $P = 5 \times 10^{-9}$), but substitutions along terminal branches are uniformly distributed among first, second and third positions (χ^2 test, $P = 0.2$).

The operation of purifying selection on internal branches, but not on terminal ones, is consistent with a model in which the tree's internal branches trace the evolution of active, functional lineages, whereas external branches are generated by neutrally evolving copies. This conclusion is supported by the frequency distribution of deletions, all of which are unique. Presumably, deletions in the reverse transcriptase are incompatible with transposition and are eliminated by purifying selection in active lineages. Furthermore, the observed positive and monotonic correlation (sign test, $P = 0.043$) (Fig. 3c) between the number of terminal-branch substitutions and the number of deletions in a sequence indicates that deletions and terminal-branch substitutions have accumulated after transposition. This relation is expected because both deletions and substitutions should accumulate with time. An alternative explanation is that *Helena* reverse transcriptase generates substitution errors at an extremely high rate (up to 7×10^{-2} mutations per nucleotide per cycle) and also produces multiple internal deletions, both of which are unlikely^{6-8,15}.

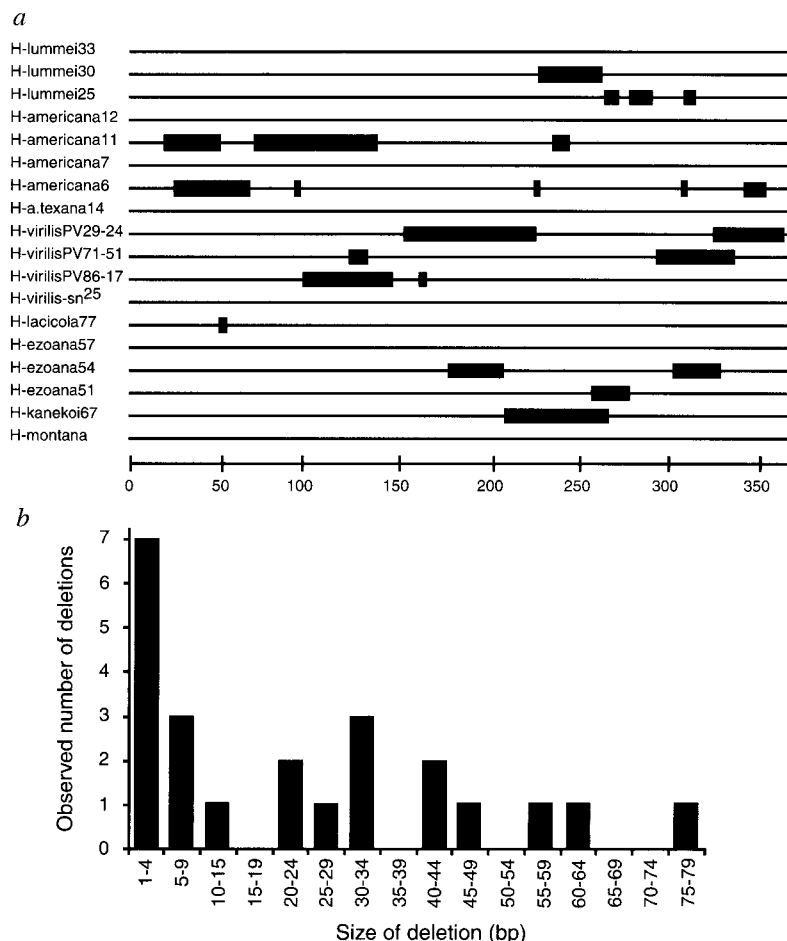


FIG. 2 Location and size of deletions in *Helena*. **a**, Location of deletions in the 18 aligned sequences of *Helena*; deletions are shown as bars. We have not observed any internal insertions relative to the consensus of the sequenced region; the only insertion we found is located at the very end of one of the sequences (H-*americana* 6) and is accompanied by a partial duplication of the primer sequence. **b**, Size distribution of the deletions.

The positive and monotonic relationship between the number of deletions and the number of terminal-branch substitution allows us to assess the deletion rate relative to the rate of nucleotide substitution. Because we cannot assume that the numbers of terminal-branch substitutions and deletions are drawn from a bivariate normal distribution, we use the approach of maximum likelihood to estimate the regression of number of terminal-branch substitutions (corrected for the length of each sequenced copy) on the number of deletions (Fig. 3c, legend), which gives us the relative rate of 0.16 deletions per substitution (the 95% confidence interval is 0.09–0.26 deletions per substitution). (Note that this estimate of the deletion rate is conservative, because a small proportion of the terminal-branch substitutions may have occurred when the sampled element was still actively transposing and under purifying selection. A slight, albeit non-significant, excess of third position substitutions on the terminal branches supports this interpretation.)

To compare the observed rate of accumulation and the size of deletions in *Helena* with that in mammals, we have analysed a dataset of human and murine processed pseudogenes compiled by Graur *et al.*¹⁶. Deletions in *Helena* are 3.3 (1.6 to 6.6) times more frequent relative to the rate of nucleotide substitutions, and are on average more than 7 times larger (24.3 ± 23.1 bp compared with 3.2 ± 4.6 bp) than deletions in the mammalian processed pseudogenes. Such a high rate of deletion and the large size of those deletions combine to eliminate DNA 25 times faster in 'dead-on-arrival' copies of *Helena* than in mammalian pseudogenes relative to the nucleotide substitution rate.

Such a high incidence of deletions could be a result of an increased rate of DNA deletion in *Drosophila* or, alternatively, of direct selection for a smaller genome. The latter model predicts a correlation between the total length of deleted DNA and the number of terminal branch substitutions, which is not supported by our data.

Is such high rate of DNA loss a general feature of *Drosophila* or merely a property of a particular stretch of 363 bp of DNA in a particular transposable element? Nothing about the position of deletions or their surrounding sequences suggests that the deletions were generated by a mechanism specific to the sequence of *Helena*. There are no hotspots of deletions: all deletions are unique and evenly distributed along the sequence. The breakpoints in about half (11 out of 23) of the deletions contain short direct repeats from 2–7 bp in length. In each case, the repeat is different, and there is no correlation between the presence of direct repeats and the length of the deletion. The presence of repeats suggests that at least some of the deletions were generated by a homology-dependent mechanism, such as recombination or DNA replication slippage^{17,18}, but, because an arbitrary sequence would contain a large number of random 2–7-bp repeats (for instance, a 1,000-bp stretch of DNA would be expected to have more than 2,000 2-bp direct repeats separated by 0–75 bp), any random stretch of DNA would be prone to multiple deletions by such a mechanism.

If such a high rate of large deletions is a general feature in *Drosophila*, this would have profound consequences for *Drosophila* genome evolution. We expect, based on the estimates of the neutral nucleotide substitution rates in *Drosophila* and mammals¹⁹, that a pseudogene in *Drosophila* would lose its DNA much more quickly than a mammalian pseudogene (average time to loss of 50 per cent of the DNA is 11.8 million years in *Drosophila*, compared with over 800 million years in mammals) and will quickly become unrecognizable by both DNA hybridization and polymerase chain reaction. Quick elimination of pseudogenes would affect the steady-state number of pseudogenes in the genome of any *Drosophila* species and reduce the probability of detecting a homologous pseudogene in diverse species of *Drosophila*. It would also explain why all studied putative pseudogenes in *Drosophila* have been found not to evolve in a strictly neutral fashion^{2–5}. Purifying selection, gene conversion or some other mechanism must have preserved their integrity, since true pseudogenes would be obliterated by multiple large deletions and could not be detected.

Differences in deletion rate may also contribute to the divergence in genome size among taxa, the so-called 'C-value paradox'. Two reports^{20,21} find a positive correlation between genome size and intron size in a variety of taxa. In addition, the reduction in the intron size in birds, whose genome size is smaller than that of other tetrapods, has been inferred to be due to multiple separate deletions scattered along the introns²¹. It is noteworthy that pseudogenes are much rarer in birds than in mammals¹. These results argue that differences in genome size among related organisms may be determined primarily by the variation in the

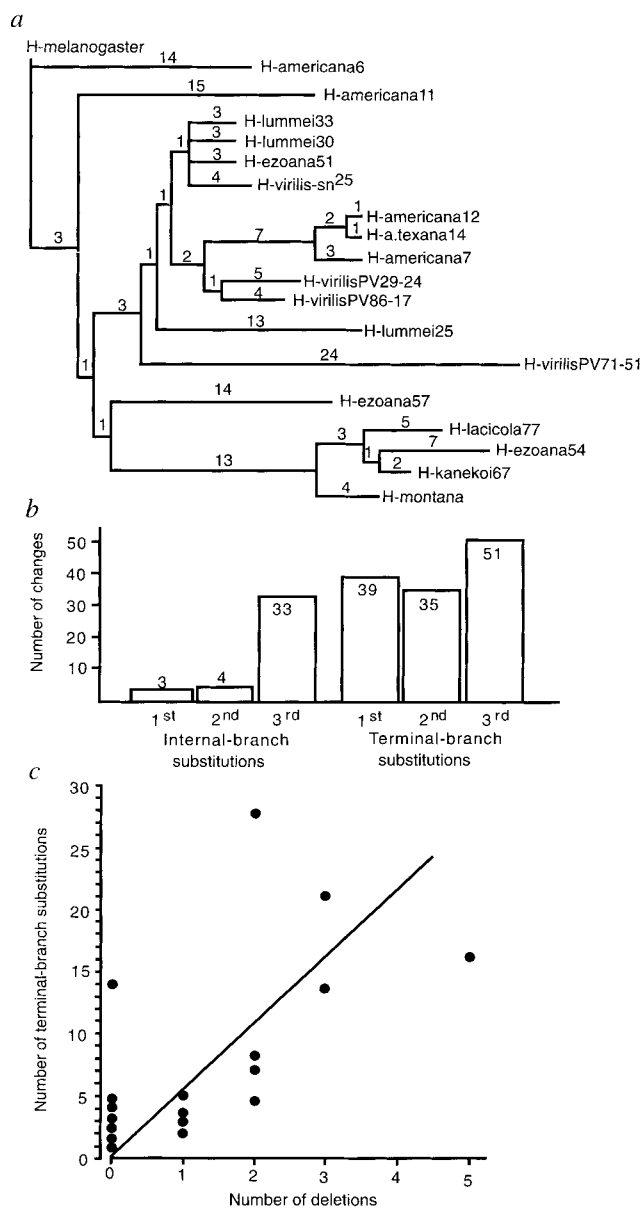


FIG. 3 Phylogenetic analysis. *a*, Maximum parsimony tree using all characters in the nucleotide alignment at equal weight. Each sequence is labelled with the species name and a unique number (see Methods). A *D. melanogaster Helena* sequence was used to root the tree. The number of unambiguous substitutions is shown above each branch. This tree is one of 75 equally parsimonious trees which differ in the placement of the branches *H-virilis* PV71-51, *H-americana* 6, *H-americana* 11 and *H-lummei* 25. Different placements of these branches introduce minor changes into the distribution of substitutions along the internal and the terminal branches. *b*, Distribution of substitutions along the internal and terminal branches. *c*, Relationship between the number of deletions and the number of terminal-branch substitutions (corrected for the length of each sequence). The solid line is the maximum-likelihood regression line.

genome-wide deletion rate, and not, for instance, by different rates of insertion of transposable elements. The wide phylogenetic distribution of non-LTR retrotransposable elements¹⁰ may permit the application of the approach used here to other taxa, to determine whether or not there is a correlation between deletion rate and genome size. □

Methods

Cloning. A single strain was used to generate *Helena* sequences for each species. Each copy is designated with a unique number and the name of the species from which it was isolated. *H-virilis-sn*²⁵, *H-montana* and *H-italmelanogaster* sequences were cloned directly using λ libraries. The rest were obtained by TA cloning (Invitrogen) of the products of the PCR reaction using primers T⁺ (5'-CAACAACGCGGTGGCTCAAC-3') and T⁻ (5'-GATT-TAATGCGGTGGTCTT-3'). These primers amplify a 363-bp stretch of DNA from the very 3' end of the putative *Helena* reverse transcriptase. The sequences *H-virilis* PV71-51, *H-virilis* PV86-17 and *H-virilis* PV29-24 were obtained using the respective P1 clones as template (for instance, DNA of the P1 clone PV71-51 was used to amplify *H-virilis* PV71-51); the rest were obtained using genomic DNA as template. Cloning in λ libraries, DNA hybridization and other standard protocols were carried out as described¹¹.

Sequencing. Preparation of the sequencing templates used a transposon-facilitated protocol²². DNA sequencing was carried out on a ABI373A automated DNA sequencer (Perkin Elmer) with the Taq Cycle Sequencing Dye-Primer or DyeDeoxy Terminator kits. All sequences have been deposited in the GenBank data base under accession numbers U65653 and U62715–U62731.

Phylogenetic analysis. Sequence alignment was done with the aid of Sequencher 2.0 (GeneCodes). Maximum parsimony analysis was carried out using the PAUP¹² software package. It used all the characters in the nucleotide alignment at equal weight. Deletions were treated as missing data. We also used the MacClade²³ software package to aid with analysis and manipulation of the *Helena* gene trees.

Statistical methods. Maximum-likelihood estimate of relative rate of deletions versus nucleotide substitutions was found under the assumptions that each sequence, upon transposition, has no deletions or unique substitutions, that rates of deletions and substitutions are constant in time, and that, at any given time, the number of deletions and the number of substitutions follow a Poisson distribution. The confidence limits were found using the χ^2 approximation of the log-likelihood ratio. The positive correlation between the number of terminal branch substitutions and the number of deletions was ascertained using a sign test.

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CORRESPONDENCE and requests for materials should be addressed to D.A.P. (e-mail: dpetrov@oeb.harvard.edu). Nucleotide alignments and information on statistical methods used in this study are available on the World Wide Web at <http://www.oeb.harvard.edu/hartl/lab/dmitri.html>, or on request.

Amelioration of the dystrophic phenotype of *mdx* mice using a truncated utrophin transgene

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DUCHENNE muscular dystrophy (DMD) is a severe, progressive muscle-wasting disease that causes cardiac or respiratory failure^{1,2} and results in death at about 20 years of age. Replacement of the missing protein, dystrophin, using myoblast transfer in humans or viral/liposomal delivery in the mouse DMD model is inefficient and short-lived^{3,4}. One alternative approach to treatment would be to upregulate the closely related protein, utrophin^{5,6}, which might be able to compensate for the dystrophin deficiency in all relevant muscles^{7,8}. As a first step to this approach, we have expressed a utrophin transgene at high levels in the dystrophin-deficient *mdx* mouse. Our results indicate that high expression of the utrophin transgene in skeletal and diaphragm muscle can markedly reduce the dystrophic pathology. These data suggest that systemic upregulation of utrophin in DMD patients may lead to the development of an effective treatment for this devastating disorder.

A truncated utrophin transgene was modelled on the Becker dystrophin transgene, which has been shown to correct the dystrophic phenotype of *mdx* mice^{9,10} (Fig. 1A). To generate high levels of muscle expression, the utrophin transgene was driven by the human skeletal α -actin (HSA) promoter (Fig. 1B). Several transgenic lines expressing the utrophin transgene were generated with differing levels of transgene expression. Immunoblot analysis of muscle samples from transgenic lines demonstrating high-level expression are shown in Fig. 1C. The multiple fainter bands probably result from the proteolytic breakdown of the highly expressed transgene product¹¹. Line 347 also shows weak expression of the transgene in the heart. Line 261 contains, but does not express, the transgene. Further analysis of the F-3 line shows no evidence of transgene expression in heart, brain, kidney, lung, liver, intestine, skin or pancreas. To demonstrate that the utrophin transgene localized to the sarcolemma, immunofluorescence of skeletal-muscle sections from the F-3 line was performed using antibodies specific to utrophin and dystrophin. The sarcolemmal localization pattern of dystrophin (Fig. 1D, a) and the utrophin transgene (Fig. 1D, b) in tibialis anterior (TA) muscle sections demonstrate that they can colocalize to the sarcolemma *in vivo*. In contrast with the transgenics, the normal localization of utrophin in adult skeletal muscle is exclusively at the neuromuscular and myotendinous junctions and in the capillaries and nerves^{12,13}.

The limb muscles of the dystrophin-deficient *mdx* mouse only develop atrophy and weakness late in life. But histological and physiological analysis reveals several muscle defects in common with DMD patients, including muscle-fibre degeneration, giving rise to a dramatic elevation of serum creatine kinase (CK) level and evidence of massive myofibre regeneration, with most fibres having centrally located nuclei¹⁴. Thus changes in the levels of serum CK and numbers of centralized nuclei have been used to monitor the pathology of the muscle in several transgenic lines expressing dystrophin transgenes in *mdx* mice^{9,10,11,15}. Male transgenic F-3 mice carrying the utrophin transgene were crossed with dystrophin-deficient female *mdx* mice. The resultant offspring were analysed (Fig. 2a) at approximately 5 weeks of age when the skeletal muscle is still undergoing rounds of degeneration and regeneration. The CK levels of male transgenic *mdx* mice (utro-tg

mdx) had fallen to approximately a quarter of that of non-transgenic *mdx* male littermates (*mdx*). Females, whether transgenic or not, have roughly normal levels of serum CK. The reduction in the serum levels of CK in the male utrophin transgenic *mdx* littermates signifies a change in the muscle pathology of these mice, and implies that a marked decrease in muscle degeneration has occurred. The contrast in numbers of

centralized nuclei in frozen sections from the soleus and TA muscle of transgenic (utro-tg *mdx*) and non-transgenic male *mdx* mice is shown in Fig. 2b. The numbers of centrally nucleated myofibres is markedly reduced in the two muscle types examined, showing that the amount of fibre regeneration is decreased. The difference in numbers of central nuclei between the utrophin transgenic *mdx* TA (~10%) and soleus (~30%) is probably

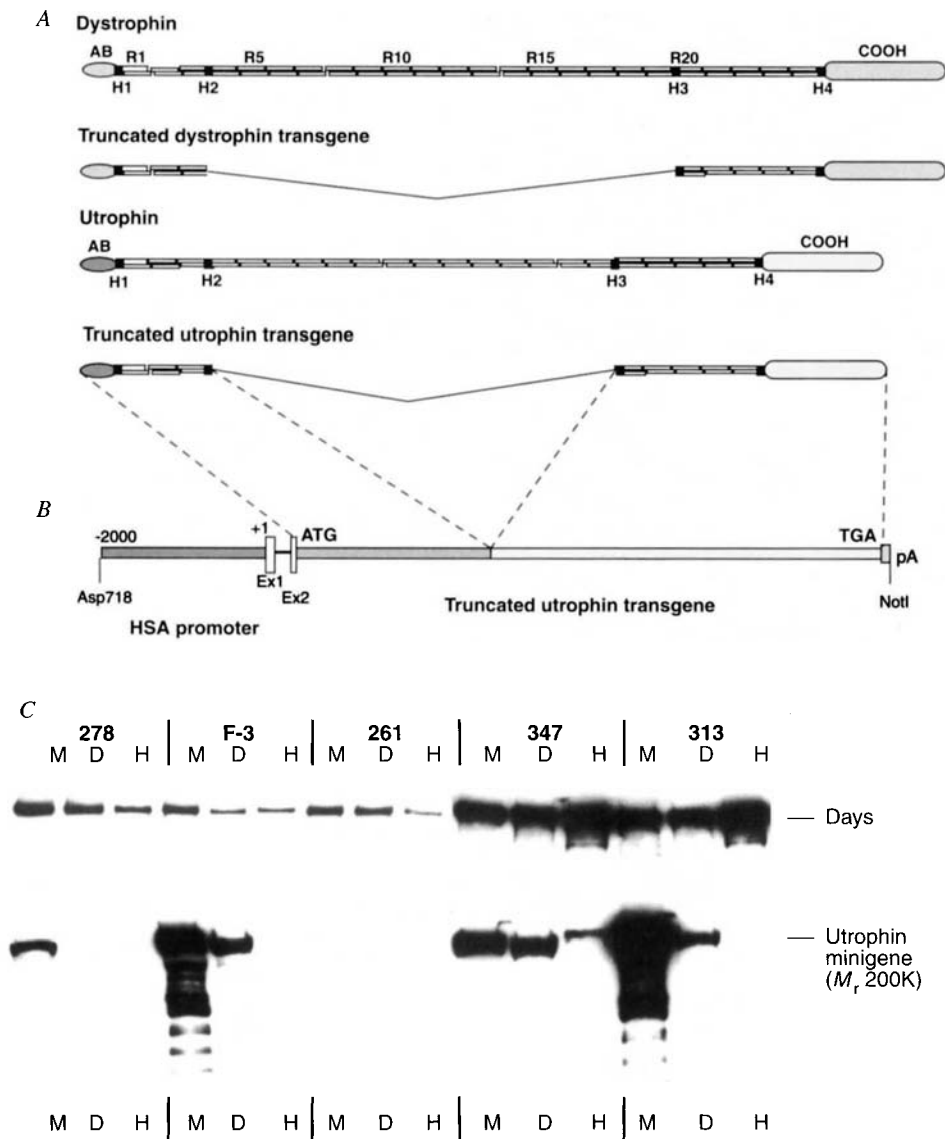
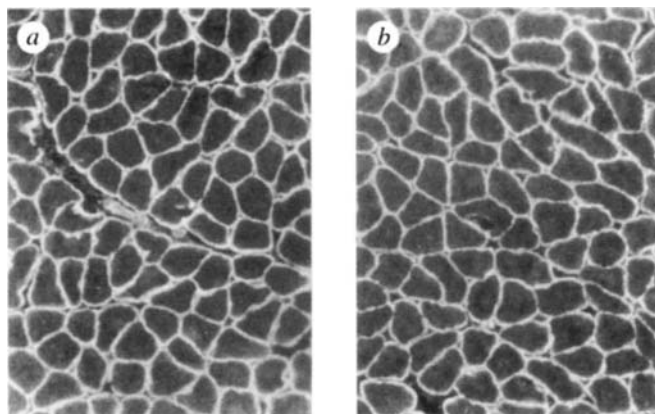


FIG. 1 Utrophin transgene construction and expression. **A**, Representation of dystrophin, utrophin and the two truncated transgenes. The spectrin-like repeats (R, each repeat depicted by the double rectangle), hinge sites (H), actin-binding (AB) and C-terminal-binding (COOH) domains. **B**, Utrophin transgene vector. For details of construction, see Methods. **C**, Immunoblot of muscle from utrophin-transgenic lines (278, F-3, 347, 313) and non-expressing line (261). M, skeletal muscle; H, heart; D, diaphragm. **D**, Immunohistochemistry of tibialis anterior muscle sections from the F-3 transgenic line showing dystrophin (**a**) and utrophin (**b**) sarcolemmal immunostaining. Magnification, $\times 100$.

D



explained by the fact that the human skeletal α -actin promoter is expressed at lower levels in the slow-twitch fibres which essentially populate the soleus muscle than in the fast-twitch fibres of the TA (L. Levitt and E. Hardeman, personal communication). This observation implies that the levels of utrophin transgene are important for amelioration of the muscle phenotype.

Dystrophin is normally associated with a large oligomeric protein complex, the dystrophin protein complex (DPC),

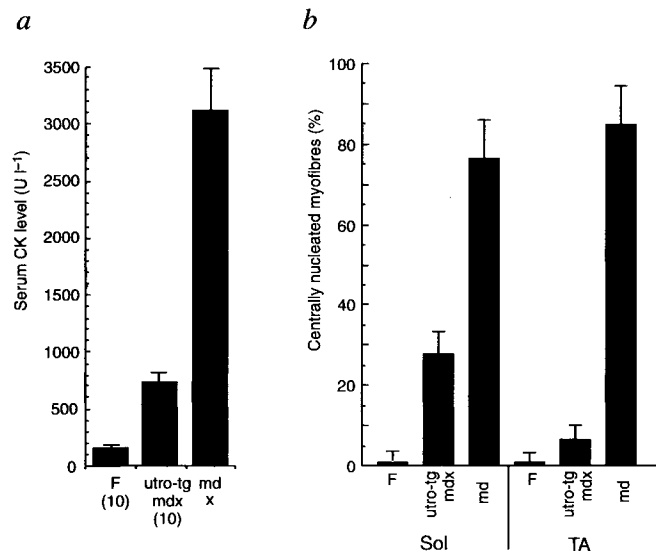


FIG. 2 Decrease in serum CK levels and centralized myofibres in transgenic *mdx* mice. *a*, Serum CK levels from male *mdx* (*mdx*), male utrophin transgenic *mdx* (*utro-tg mdx*) and heterozygous females (*F*). Female heterozygotes are not significantly different from wild type, and so can be used as normal controls. The number of mice in each group is given in parentheses and the s.e.m. is shown. *b*, Proportion of myofibres containing centralized nuclei. The s.e.m. is shown.

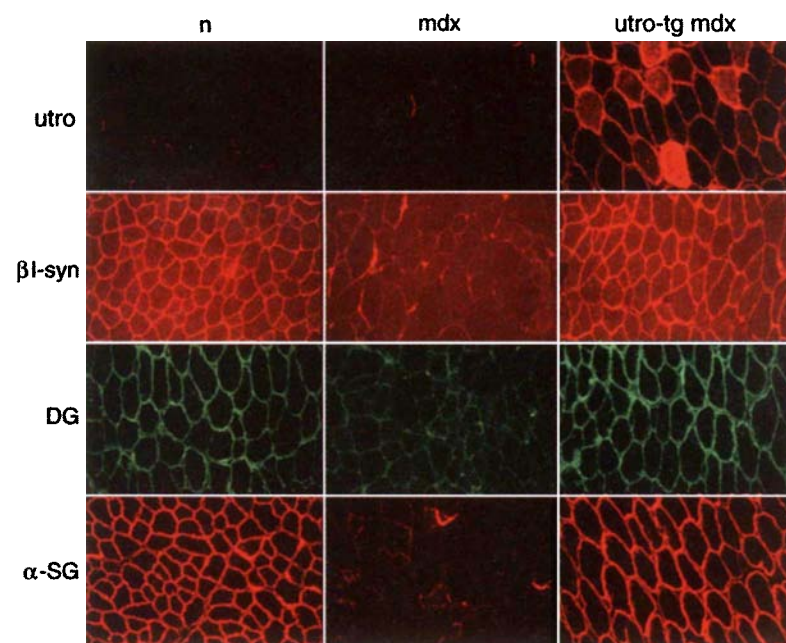


FIG. 3 Increase in DPC staining at the sarcolemma of transgenic *mdx* mice. Immunohistochemistry of utrophin and components of the DPC in TA muscle of normal (*n*), male *mdx* (*mdx*) and male transgenic *mdx* (*utro-tg mdx*) using antibodies specific to utrophin (*utro*), β 1-syntrophin (β 1-syn), α/β -dystroglycan (*DG*) and α -sarcoglycan (α -SG). Magnification, $\times 100$.

embedded in the carcolemma^{12,16}. Loss of dystrophin in DMD patients and *mdx* mice also results in a dramatic loss of sarcolemmal DPC¹⁷. In transgenic *mdx* mice expressing the full-length and truncated dystrophin transgenes, re-establishment of components of the DPC at the sarcolemma is an important marker for the restoration of muscle strength by dystrophin transgenes^{9,10,11,15}. The results of immunostaining for components in the DPC in TA muscle from normal (*n*) mice, male *mdx* mice (*mdx*) or *mdx* mice expressing the utrophin transgene (*utro-tg mdx*) are shown in Fig. 3. Sarcolemmal staining of all myofibres by utrophin-specific antibody was seen in utrophin transgenic *mdx* muscle. But in the normal and non-transgenic *mdx* mice there is virtually no sarcolemmal staining apart from neuromuscular junctions and regions likely to contain regenerating fibres. In all cases using polyclonal antibodies specific to β 1-syntrophin, α/β -dystroglycan, α -sarcoglycan and γ -sarcoglycan (data not shown), there was a notable increase in the staining at the sarcolemma of utrophin transgenic *mdx* TA muscle, indicating an elevation in sarcolemmal bound components of the DPC. The level of sarcolemmal staining of the utrophin transgenic *mdx* mice appears to be greater than that seen in the normal TA, suggesting that the number of dystrophin protein complexes at the sarcolemma is limited to some degree by the amount of dystrophin (or utrophin) present in the myofibres. This is a similar phenomenon to that observed in *mdx* mice expressing very high levels of a dystrophin transgene¹¹. The increase in sarcolemmal staining of these components in the soleus muscle of transgenic *mdx* mice is greater than the non-transgenic *mdx* males but not as elevated as in the TA (data not shown). This result again implies that increased utrophin transgene expression correlates with an increase in sarcolemmal bound DPC.

Analysis of the *mdx* diaphragm has shown that this muscle exhibits a continued pattern of degeneration, fibrosis and functional deficit throughout the lifespan of the *mdx* mouse comparable to that of DMD skeletal muscle¹⁸. Thus, for utrophin to be capable of replacing dystrophin, overexpression of utrophin in this muscle has to alter the pathology in a similar way to that demonstrated for the dystrophin transgenic *mdx* mice^{9,10,11,15}.

Immunostaining of diaphragm sections using a utrophin antibody demonstrates the sarcolemmal localization of the utrophin transgene expressed in the transgenic *mdx* mouse (*utro-tg mdx*) compared to normal and *mdx* mice (Fig. 4*a*). The re-establishment of α -sarcoglycan at the sarcolemma of the transgenic *mdx* diaphragm at levels similar to the normal diaphragm is shown in Fig. 4*a*. Sarcolemmal staining of the transgenic *mdx* diaphragm is similar to normal using antibodies specific to α/β -dystroglycan and γ -sarcoglycan (data not shown). Thus, as in skeletal muscle, expression of the utrophin transgene in diaphragm relocates the DPC to the sarcolemma. Histological analysis of haematoxylin and eosin-stained sections of *mdx* diaphragm shows extensive regions of fibrosis, cellular infiltration and variable myofibre size containing centralized nuclei (Fig. 4*b*, *mdx*). But the utrophin transgenic diaphragm looks essentially the same as normal (Fig. 4*b*, 'n') with no necrosis, regular myofibre size and virtually no centralized nuclei (Fig. 4*b*, *utro-tg mdx*). In the *mdx* diaphragm, even in regions which have no necrosis and so appear more histologically normal, on higher magnification the myofibres are still of variable size, often containing centralized nuclei, which is indicative of continual regeneration (Fig. 4*c*, *mdx*). In the utrophin transgenic diaphragm, even at higher magnification, the whole muscle appears normal (Fig. 4*c*, *utro-tg mdx*). A return to normal histology and establishment of the DPC are two important observations, as seen with the dystrophin-transgenic

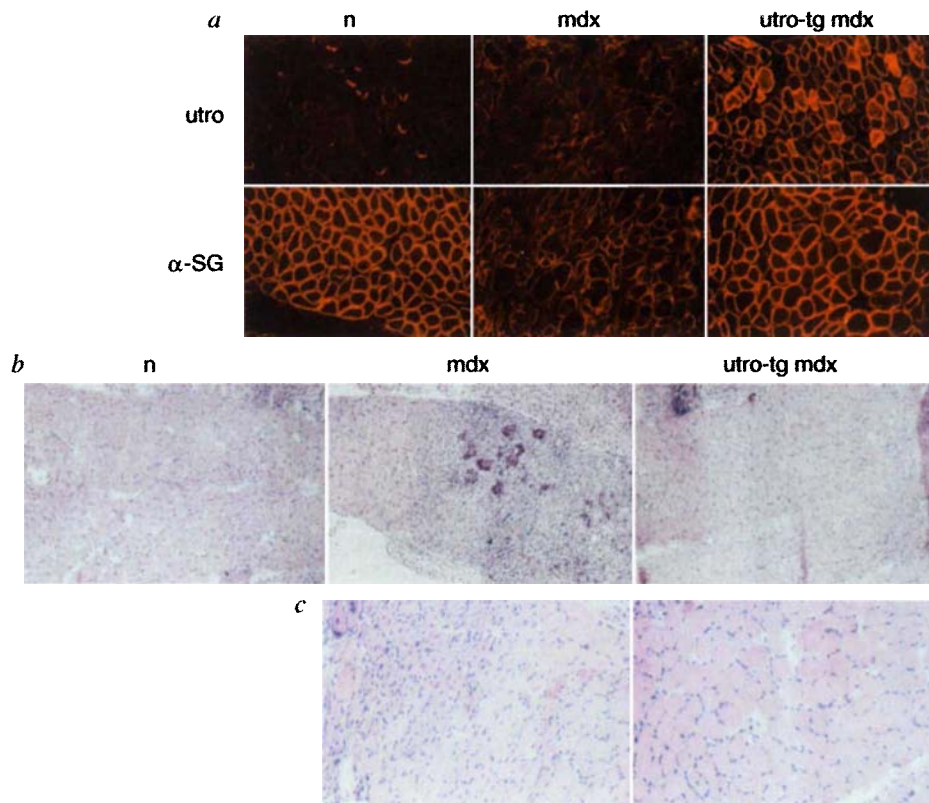


FIG. 4 Immunostaining and histological analysis of normal, *mdx* and utrophin transgenic *mdx* diaphragm. **a**, Immunostaining of diaphragm sections from normal (*n*), *mdx* (*mdx*) and transgenic *mdx* (utro-tg-*mdx*) stained with antibodies against utrophin (utro) and α -sarcoglycan (α -SG). Magnification, $\times 100$. **b**, Haematoxylin and eosin staining of normal (*n*), *mdx* (*mdx*) and transgenic *mdx* (utro-tg *mdx*) diaphragm sections. Magnification, $\times 100$. **c**, Higher magnification ($\times 200$) of *mdx* diaphragm (*mdx*) in areas with no obvious fibrosis/cellular infiltration still show evidence of continual regeneration, as seen by the variable fibre size and presence of centralized nuclei.

mice^{9,10,11,15}, which predicts a major recovery of the utrophin-transgenic diaphragm from a dystrophic phenotype.

We have demonstrated a significant decrease in the dystrophic muscle phenotype of *mdx* mice by expressing a utrophin transgene at high levels in the skeletal muscle and the diaphragm. These results strongly suggest that utrophin can replace dystrophin *in vivo*. This implies that the use of small molecules which increase the normal utrophin muscle expression to compensate and therefore alleviate the consequences of a lack of dystrophin, is a promising area for DMD therapy. It remains to be seen if such compounds can be identified. However this approach would potentially target all muscles and thus prolong life by conserving the respiratory and cardiac muscles. Utrophin is expressed in many tissues so a generalized upregulation may not have detrimental side effects. The normal mice expressing the utrophin transgene at high levels seem to suffer no deleterious affects in their skeletal and diaphragm muscles. The side effects, if any, have yet to be demonstrated in other tissues. A precedent for such a gene-therapy approach using butyrate to upregulate fetal haemoglobin is having success in clinical trials of sickle-cell disease^{19,20}. Only 20–30% of the wild-type levels of dystrophin are required to reduce significantly the dystrophic phenotype in *mdx* mice^{9,20}. It will be interesting to determine whether similar level of utrophin will be adequate to compensate for dystrophin loss. Furthermore, as utrophin is normally expressed in all tissues including muscle, the use of this utrophin transgene rather than a dystrophin transgene in conventional gene-therapy approaches using viruses or liposomes may avert any potential immunological responses against the transgene. □

Methods

Transgene construction and microinjection. The amino- and carboxy-terminal portions of utrophin were cloned as polymerase chain reaction (PCR) products using overlapping complementary DNAs as template, then ligated together in-frame to produce the truncated utrophin cDNA. The PCR product was then cloned into a vector containing the 2.2-kilobase human skeletal α -

actin (HSA) promoter and regulatory regions^{21,22} and SV40 large T poly (A) site. The cloning sites were such that the transgene was located near the beginning of the second HSA untranslated exon and the Asp 718/NotI sites were used to liberate the complete fragment. Transgenic mice were generated by microinjection of the purified HSA transgene insert into the pronucleus of F₂ hybrid oocytes from C57BL/6x129/Ola parents²³. Positive transgenic mice were identified by Southern blotting using a probe to the central part of the utrophin transgene. Several founder F₀ males were bred to generate more offspring for analysis and breeding.

Protein analysis. Total muscle extracts were prepared by homogenization in 1 ml extraction buffer (75 mM Tris, pH 6.8, 3.8% SDS, 4 M urea, 20% glycerol, 5% β -mercaptoethanol) then heated to 95°C for 5 min. Usually 50 μ g of total protein (quantified using Biorad DC protein assay kit) was loaded onto 6% polyacrylamide gels and transferred to nitrocellulose. Utrophin transgene expression was detected using a 1/200 dilution of mouse anti-utrophin monoclonal antibody (MANCH07; ref. 24) and visualized using anti-mouse IgG-POD and chemiluminescence (Boehringer). For sectioning, skeletal muscle samples were removed and immersed in OCT compound (BDH) and frozen in liquid-nitrogen-cooled isopentane. The diaphragm was removed, cut in half, then rolled longitudinally and sandwiched between ox liver to facilitate orientation and easier sectioning. The sandwich was then frozen. Immunostaining of unfixed 8 μ m cryosections was performed by blocking the sections in 10% heat-inactivated fetal calf serum in 50 mM Tris, 150 mM NaCl, pH 7.5 (TBS), then the primary antibody diluted in TBS added and incubated for 1 h at room temperature. The slides were washed four times in TBS for 5 min each, then incubated for a further hour at room temperature with conjugated second antibody diluted in TBS. Finally, the slides were washed as before, mounted with VectaShield (Vector) and photographed using a Leica DMRBE microscope and photomicrograph system.

Antibodies used for immunofluorescence. Antibodies were used at the following dilutions: polyclonal rabbit against utrophin (G3, 1/25), dystrophin (P6 (ref. 25), 1/400), β 1-syntrophin (syn35, 1/50) α -sarcoglycan²⁶ (1/5); goat polyclonal against α / β -dystroglycan (FP-B²⁷, 1/10); FITC conjugated secondary antibody to goat (Sigma) and Cy3 conjugated secondary antibody to rabbit (Jackson) were diluted 1/50 and 1/200, respectively.

Creatine kinase assay. Serum CK levels from mice 4–5 weeks old generated from four F₃ litters resultant from a male transgenic mouse crossed with female *mdx* were assayed. The tail tips were cut off and DNA prepared for Southern blotting to establish the transgenic status of each mouse. Blood was collected simultaneously, allowed to clot, and serum was removed. Serum CK levels were measured using the Boehringer NAC-CK kit and 5 μ l of serum. The rate was averaged over 4 min and calculated as U l⁻¹.

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Inhibition of myocardial endothelin pathway improves long-term survival in heart failure

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Occlusion of the diseased coronary artery in humans causes acute myocardial infarction, survivors of which have a high risk for the development of chronic heart failure¹. Cardiac myocytes and vascular endothelial cells produce endothelin-1 (refs 2–4), which increases the contractility of cardiac muscle and of vascular smooth muscle cells^{2,3,5}. Endothelin-1 also exerts long-term effects such as myocardial hypertrophy, and causes cellular injury in cardiac myocytes^{3,5–7}. Production of endothelin-1 is markedly increased in the myocardium of rats with heart failure, and acute application of an endothelin-receptor antagonist decreases myocardial contractility in such rats, indicating that myocardial endothelin-1 may help to support contractility of the failing heart⁸. But we report here that the upregulated myocardial endothelin system may contribute to the progression of chronic heart failure, because long-term treatment with an endothelin-receptor antagonist greatly improved the survival of rats with chronic heart failure. This beneficial effect was accompanied by significant amelioration of left ventricular dysfunction and prevention of ventricular remodelling, in which there is usually an increase in the ventricular mass and cavity enlargement of the ventricle.

We used rats with myocardial infarction caused by coronary ligation as a model^{9,10} of experimental heart failure. We first investigated the effects of long-term (12 weeks) administration of an antagonist of the endothelin-A (ET_A) receptor, known as BQ-123 (ref. 11), on survival and cardiac dysfunction in these rats. Administration of BQ-123 almost doubled the number of rats with heart failure that survived (HF rats; Fig. 1); the 12-week survival of rats treated with BQ-123 was 85%, compared with only 43% for control rats treated with saline.

We next examined the histological features of hearts from surviving animals. HF rats treated with saline show severe ventricular remodelling (compare Fig. 2a and b): the infarcted area of the surviving myocardium is replaced by fibrous scar tissue, there

is a progressive increase in the ventricular mass of the surviving myocardium, and the ventricular cavity is enlarged. Long-term administration of BQ-123 prevents these changes (compare Fig. 2b and c), which in human patients are associated with an increase in cardiovascular-related death¹. In addition, the cardiac myocyte hypertrophy seen in HF rats (compare Fig. 2d and e) is effectively prevented by long-term BQ-123 treatment (compare Fig. 2e and f). Although appropriate myocardial hypertrophy of the surviving myocardium is thought to compensate for myocardial loss in the infarcted area, excessive hypertrophy of the myocardium is believed to be maladaptive and to be associated in part with dysfunction of the surviving myocardium¹² and unfavourable ventricular remodelling. The reduction in ventricular remodeling induced by BQ-123 treatment therefore appears to partly contribute to the improvement in the long-term mortality of HF rats.

We next made haemodynamic measurements to investigate cardiac function in the surviving animals. When compared with sham-operated (SO) rats, HF rats treated with saline consistently showed signs of decreased cardiac function; a lower first derivative of left-ventricular pressure ($LV + dp/dt_{max}$), a parameter of cardiac contractility that is decreased by chronic heart failure (Fig. 3a); a higher left-ventricular end-diastolic pressure (LVEDP), a parameter that is increased by chronic heart failure (Fig. 3b); a raised right-ventricular systolic pressure (RVSP), an indicator of pulmonary hypertension (Fig. 3c); and an increased central venous pressure (CVP) (Fig. 3d). Long-term administration of BQ-123 improved each of these conditions, which are all normally associated with heart failure. In normal rats, treatment with the same dose of BQ-123 for 4 weeks did not cause any haemodynamic alteration or morphological change in cardiovascular tissue (our published observations).

The production of endothelin-1 (ET-1) is increased in rats with heart failure⁸, but it is not known whether myocardial or endo-

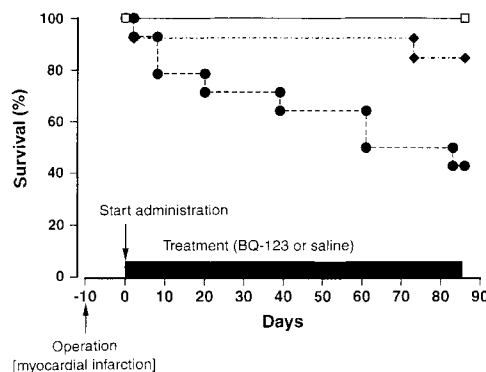


FIG. 1 Survival curves of heart failure rats treated with saline (●) or BQ-123 (◆) and of sham-operated (SO) rats treated with saline (□). Treatment started 10 days after coronary artery ligation. The day of the operation is indicated by an arrow. The number of rats in each group was: □, $n = 8$; ●, $n = 14$; ◆, $n = 13$. $P < 0.01$.

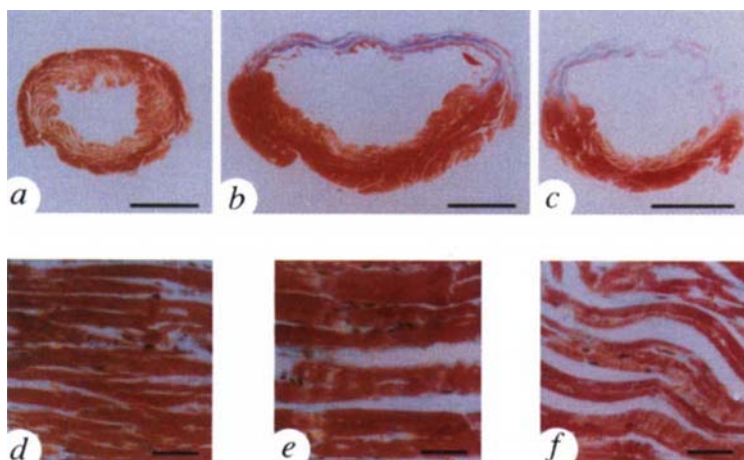


FIG. 2 Macroscopic (a–c) and microscopic (d–f) histological analysis using Masson trichrome staining in the left ventricle of surviving rats. a, d, SO + saline; b, e, HF + saline; c, f, HF + BQ-123 (see Methods for notation). Results similar to those shown here were observed for all surviving animals analysed. Scale bars: a–c, 5 mm; d–f, 50 μ m.

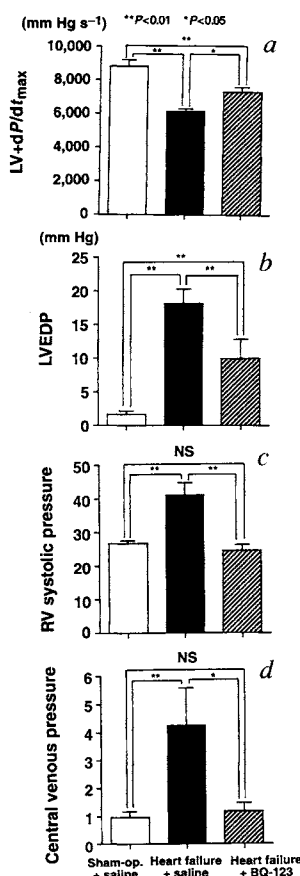


FIG. 3 The effects of long-term BQ-123 administration on a, the peak positive first derivative of left ventricular pressure (LV + dP/dt_{max}); b, left ventricular end-diastolic pressure (LVEDP); c, right ventricular (RV) systolic pressure; and d, central venous pressure in surviving rats. Bars represent SO rats treated with saline (white), HF rats treated with saline (black) or BQ-123 (hatched). The number of rats in each group was 8, 6 and 11, respectively. *, $P < 0.05$, **, $P < 0.01$; NS, not significant.

thelial cells of the coronary artery are responsible for this increase. We therefore investigated whether the intensity of ET-1 immunoreactivity is altered in these cells using rats that developed chronic heart failure three weeks after surgery. Immunohistochemistry revealed that ET-1 immunoreactivity in cardiac myocytes was higher in HF rats than it was in SO rats; there was no difference in the intensity of staining in coronary arteries between the two groups (compare Fig. 4a, c with b, d). The amounts of precursor (prepro)-ET-1 messenger RNA (Fig. 5a) and ET-1 protein (Fig. 5b) in the left ventricle were raised in HF rats compared with SO rats; the expression of prepro-ET-1 mRNA in the kidney did not differ between the two groups (Fig. 5a). These results are consistent with our previous findings⁸. We also observed that expression of prepro-ET-1 mRNA in the left ventricle increased as chronic heart failure progressed, as quantified by densitometric analysis⁸. The ratio of prepro-ET-1 mRNA to GAPDH (glyceraldehyde-3-phosphate dehydrogenase, an internal control)

mRNA in HF rats was higher than in SO rats by 1.4-, 2.5-, 3.3- and 4.8-fold at 2, 3, 4 and 5 weeks after surgery, respectively. Binding of ¹²⁵I-labelled ET-1 to cardiac membranes showed that binding-site density was higher in HF rats than in SO rats ($B_{max} = 243.0 \pm 20.0$, compared with 154 ± 17.4 (mean $\pm$ s.e.m.) fmol per mg protein; $n = 5$, $P < 0.05$). Competitive displacement of bound ¹²⁵I-labelled ET-1 from cardiac membranes using BQ-123 indicates that the ratio of ET_A to ET_B receptors is 86 : 14 in HF rats, compared with 91 : 9 for SO rats. ET_A receptors are therefore dominant in both the normal and the failing rat heart. This is consistent with previous studies reporting that ET_A receptors predominate in the human heart¹³. The binding-site density of ET_A receptors was higher in HF rats than in SO rats (208.0 ± 15.2 versus 140.6 ± 17.0 fmol per mg protein; $n = 5$, $P < 0.05$), indicating that upregulation of ET_A receptors occurs in the failing heart.

BQ-123 may improve survival and ameliorate cardiac dysfunction through several mechanisms. First, as ET-1 has direct toxic effects on cardiac myocytes⁷, an increase in the production of ET-1 in HF rats may lead to myocardial injury and contribute to the progression of chronic heart failure. The positive effects of BQ-123 treatment may therefore be due to protection against the direct cardiotoxic effects of ET-1. Second, because ET-1 has a potent hypertrophic effect on cardiac myocytes^{3,5,6}, it is also likely that the upregulated ET-1 system in the heart contributes to the excessive hypertrophy of the myocardium in HF rats. BQ-123 may therefore prevent the progression of unfavourable and maladaptive¹ hypertrophy of the myocardium. Third, long-term stimulation of myocardial contractility by ET-1 in HF rats may contribute to the progression of chronic heart failure. This is consistent with observations of the failing heart in humans showing that long-term stimulation of myocardial contractility is

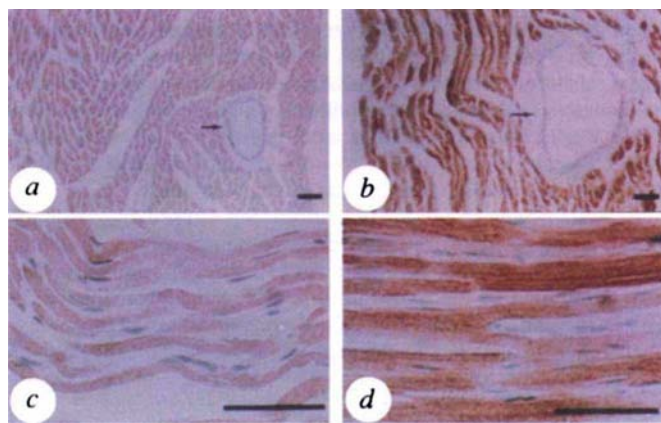
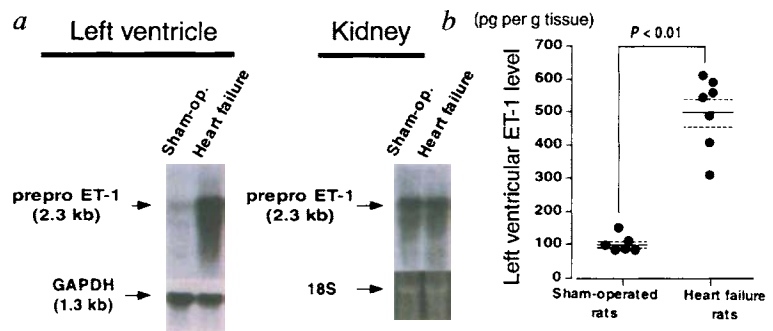


FIG. 4 ET-1 staining in the heart of a, c, an SO rat, and b, d, an HF rat 3 weeks after operation. ET-1 staining is shown in brown. Scale bar: 50 μ m. Staining similar to that shown was seen in all HF rats and 7 SO rats analysed.

FIG. 5 Expression of prepro-ET-1. *a*, Northern blot of prepro-ET-1 mRNA from the left ventricle and from the kidney of SO and HF rats. *b*, Levels of ET-1 protein in SO rats and HF rats. Solid line represents the group mean and dotted line represents the s.e.m.



harmful because it leads to increased myocardial energy utilization and ultimately to death^{14,15}. It is therefore likely that the reduction in myocardial contractility induced by BQ-123 in HF rats⁸ causes myocardial energy utilization to decrease, thereby improving the metabolic status of the myocardium. Fourth, ET-1 induces ventricular arrhythmias^{3,16}, and so it is possible that the inhibition of ET-1-induced arrhythmias by BQ-123 is beneficial. We also found that chronic treatment with a non-peptide endothelin-receptor antagonist had beneficial effects similar to BQ-123 (our unpublished data). We propose that the favourable effects of BQ-123 are due to the blockade of endothelin receptors and inhibition of ET-1-mediated downstream events. We believe that these effects are specific because the plasma concentration of BQ-123 ($3.7 \pm 0.5 \times 10^{-7}$ M, $n = 5$) does not cause any non-specific effects in cardiovascular tissues (ref. 11, and our unpublished observations).

We previously reported that prepro-ET-1 mRNA increased in the heart under some pathological conditions provoked *in vivo* by pressure overload to the left ventricle owing to aortic banding¹⁷, or to the right ventricle because of pulmonary hypertension⁶. The present study shows that the levels of ET-1 mRNA and protein are raised in HF rats and that this increase originates in cardiac myocytes and not in the coronary endothelium (Fig. 4). It is therefore likely that the excessive ET-1 produced by cardiac myocytes in HF rats acts on coronary arterial smooth muscle in a paracrine fashion, causing vasoconstriction and a subsequent reduction in myocardial blood flow in the failing heart^{2,3}. It was reported that a short (15 min) intravenous infusion of bosentan, a double ET_A- and ET_B-receptor antagonists, caused a moderate (8%) decrease in systemic blood pressure in patients with severe heart failure¹⁸. We cannot therefore exclude the possibility that the beneficial effects of chronic BQ-123 treatment in HF rats may be partly due to a reduction in afterload to the left ventricle, caused by inhibition of ET-1-induced vasoconstriction in the systemic arteries. We consider this unlikely, however, because our previous data indicate that rapid intravenous BQ-123 infusion does not affect systemic blood pressure in HF rats⁸.

We found previously that there is an increase in the plasma concentration of ET-1 in HF rats⁸ and in patients with chronic heart failure^{19,20}. The present study indicates that inhibition of the upregulated myocardial endothelin system is an important target for treatment of chronic heart failure. □

Methods

Experimental heart failure. Left ventricular free-wall myocardial infarction was induced in 6-week-old male rats according to ref. 9, as described in ref. 8. Each rat was anaesthetized with ether, a thoracotomy was performed, and the proximal portion of the left coronary artery was ligated with a silk suture. Apart from the coronary arterial ligation, sham-operated rats underwent an identical procedure. Mortality in animals with myocardial infarction was ~67% within the first 24 h; 7 d after surgery, myocardial infarction was confirmed by electrocardiography.

BQ-123 administration. BQ-123 (a gift from Masaru Nishikibe) was dissolved in saline and administered to the rats using a subcutaneously implanted osmotic minipump. As there was a possibility that the infarct size of rats with coronary artery ligation was directly affected by BQ-123 administration during the acute phase of myocardial infarction, we began BQ-123 administration 10 d after surgery. Rats were divided into 3 groups: sham-operated rats receiving

saline infusion for 12 weeks (SO + saline), rats with heart failure and receiving saline infusion for 12 weeks (HF + saline), and rats with heart failure and receiving BQ-123 infusion (7.5 mg per day per rat) for 12 weeks (HF + BQ-123). Survival data are presented as Kaplan–Meier curves, which were compared for HF rats using the log-rank test.

Haemodynamic measurements. Twelve weeks after administration of BQ-123 or saline, haemodynamic parameters for all surviving rats were determined as described⁸. Rats were anaesthetized with urethane and α -chloralose. A microtip pressure-transducer catheter (model SPC-320, Millar Instruments) was inserted into the right carotid artery. After measuring arterial blood pressure, the catheter was advanced into the left ventricle and LV + dP/dt_{max} determined. A polyethylene catheter was then inserted into the right jugular vein to measure central venous pressure and right ventricular pressure. Differences between the three groups were assessed by analysis of variance (ANOVA) with Bonferroni's multiple comparison test.

Histology. After haemodynamic measurement, hearts were excised and analysed using Masson trichrome staining. Hearts were immersion-fixed in 10% buffered formalin and the ventricles cut into transverse sections; the section from the middle of the ventricle was used for histology. Each section was cut into 3- μ m slices, stained, and examined by light microscopy.

ET-1 immunocytochemistry. Immunocytochemistry on the heart was performed as described²¹. Heart sections were stained using the biotin–streptavidin–horseradish peroxidase method for detecting ET-1. Rabbit anti-ET-1 polyclonal antibody (Peninsula) was used as the primary antibody and slides were counterstained with methyl green. The specificity of the immunoreactivity was determined by an absorption test.

Northern blot analysis. Northern blot analysis for prepro-ET-1 mRNA in the heart and kidney was done as described^{6,8}. Total RNA (15 μ g per lane) from the left ventricle (excluding scar tissue) or from the kidney was isolated and transferred onto nylon membranes, which were hybridized with a ³²P-labelled cDNA probe of a full-length insert of λ rET-1–2 as previously described^{6,8}. Expression of rat GAPDH mRNA and ethidium bromide staining of 18S ribosomal RNA were used as internal controls.

Quantification of ET-1 protein. The level of ET-1 in the left ventricle was determined as described⁸. The homogenate of the left ventricle without scar tissue was subjected to a sandwich-enzyme immunoassay (EIA) for ET-1. The sandwich EIA has been described^{6,8,22}.

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Genetic and environmental contributions to the acquisition of a motor skill

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PRACTICE, with feedback, is a fundamental variable that influences the acquisition of motor skills¹: with it, everyone improves, but some improve more than others. This simple fact has led to frequent debate over the relative importance of genetic and environmental influences on motor learning. In principle these factors could influence subjects' initial level of proficiency, their rate of improvement or their final level of attainment. The problem has been investigated using the rotary pursuit (RP) task, in which subjects learn to track a rotating target with a stylus²; this is a factorially pure task which is relatively unaffected by cognitive or verbal factors^{3,4}. Earlier studies of twins reared together^{2,5} indicated that heredity was the primary factor responsible for individual differences in motor skill. Here we have studied learning in a sample of monozygotic (MZA) and dizygotic (DZA) twins who had been reared apart. Heritability of performance was high even in the initial phase, and increased with practice. The rate of learning was also significantly heritable. We propose that the effect of practice is to decrease the effect of environmental variation (previous learning) and increase the relative strength of genetic influences on motor performance.

Performance scores (time-on-target) for the RP task were calculated in blocks of five trials, with five blocks of five trials on each of the three days of the experiment. Reliability of each block of trial measures were calculated using Cronbach's alpha coefficient⁶ and ranged from 0.92 for the first block of the first day to 0.97 for the last block of the last day. The mean time-on-target performance scores, expressed as a percentage of perfect scores (20.00 s), by trial block for MZA and DZA twin groups over all days of practice, are shown in the lower part of Fig. 1. Performance levels of the two groups are highly similar; both showing substantial improvement over the five trial blocks of the first day. Both groups also show considerable reminiscence (improvement) after the extended rest between days 1 and 2 (blocks 5–6) and days 2–3 (blocks 10–11). Patterns of variability for both groups are likewise quite similar, as shown in the upper part of Fig. 1. During the early stages of practice, levels of variability in performance are low. With practice, there is improvement for some subjects more than others, hence increases in the within-group variability with stability being found by day 3. The differences between the variances of the MZA and DZA twins were not statistically significant.

Using a repeated measures ANOVA on the RP performance scores, with day and practice-trial block as the within-subject variables, significant main effects were found for day of practice, $F_{2,174} = 628.84$, $P < 0.001$, and practice-trial block, $F_{4,172} = 36.83$, $P < 0.001$. An interaction between day and block, as shown by the differential degree of trial-block improvement as a function of day of practice (lower part of Fig. 1), was also significant, $F_{8,68} = 31.76$, $P < 0.001$.

Intraclass correlations, corrected for the effects of sex and age⁷, for MZA and DZA twin pairs over the 15 blocks of practice trials, are shown in the lower part of Fig. 2. The MZA intraclass correlations are highly regular and show a slight increase over the three days of practice. The DZA correlations are much less stable and regular. This lack of stability in the DZA correlations may, in part, be due to the smaller number of DZA pairs. The

slope of the regression line fitted to the DZA intraclass correlations for the last two days (not shown) is close to zero. These data suggest an asymptotically decreasing contribution of environmental factors as practice on the RP task continues. The consistently larger intraclass correlations for MZA compared with DZA twin sets also points to a significant genetic component of performance.

A purely environmental model was rejected for all of the 15 trial blocks, whereas a combined genetic and environmental model fitted the data at each of the 15 trial blocks. The upper curve in Fig. 2 gives the proportion of variance due to additive factors (the heritable components), together with their standard errors. The influence of heritable factors is high on the first block (0.66 ± 0.08), and remains high throughout the 15 trial blocks, ending on the last block with a value of 0.69 ± 0.08 . The highest heritability (0.74 ± 0.08) was observed for the 14th block. The influence of heritability in the first trial block (0.66) is elevated relative to the other four blocks on the first day (0.53, 0.52, 0.55, 0.52, respectively, in all cases ± 0.08). One reasonable hypothesis for this elevation is that, without previous experience, individuals rely largely on their current abilities; MZA twins share genetic influences on these abilities to a greater degree than DZA twins. With the exception of the first block of trials, there is a trend towards increased heritability across the 15 blocks. Moreover, the similarity between MZA twins across the 15 blocks of trials is indistinguishable from the block-to-block similarity of an individual's performance across time. If heritability is high, we would expect the matrix of MZA intraclass correlations among the 15 blocks not to differ significantly from the interblock correlation matrix. Whereas the MZA intrablock correlation matrix did not differ significantly from the sample interblock correlation matrix;

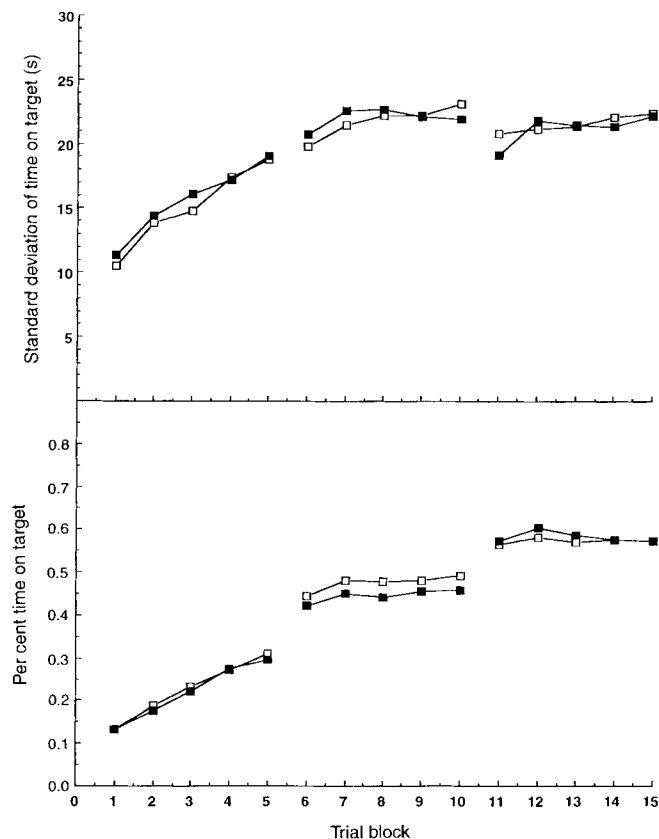


FIG. 1 Standard deviations of time-on-target (upper figure) and percentage of time on target (lower figure) for the rotary pursuit task for MZA (open squares) and DZA (filled squares) twins for each of 15 trial blocks.

$\chi^2(120, N = 160) = 92.58, P = 0.97$, the DZA intrablock correlation matrix did differ significantly; $\chi^2(120, N = 128) = 1,064.20, P = 0.0001$. Thus, MZAs perform as similarly to each other across blocks as any one individual to him or herself.

Performance on the rotary pursuit task may be examined in other ways. Table 1 shows the twin intraclass correlations, corrected for age and sex, for the slope of rotary pursuit performance on each day, and the reminiscence effect observed between days 1 and 2, and days 2 and 3. Model-fitting revealed that heritable (H) and environmental (E) factors had significant effects on performance, and the hypothesis of common parameter estimates for the three slopes ($H^2 = 0.66 \pm 0.05; E^2 = 0.34 \pm 0.03$) could not be rejected. A model including genetic and environmental effects

was also necessary to explain the two reminiscence effects and again the hypothesis of common parameter estimates ($H^2 = 0.40 \pm 0.07; E^2 = 0.60 \pm 0.04$) could not be rejected.

Our results demonstrate the significance of genetic effects for individual differences in motor skill as measured by rotary pursuit performance. Significant genetic effects were found whether performance was defined simply as per cent time-on-target, defined in terms of changes in performance over time (slope of performance), or defined as improvement after a period of rest (reminiscence effects). Our conclusions are based on a greater MZA than DZA twin resemblance for performance on each trial of practice. The simplest and most reasonable explanation for the greater MZA twin resemblance is that motor performance reflects genetic influence. With practice, increases in environmental similarity reduce twin differences more for MZA twins (who are already genetically identical) than for DZA twins, and reveal stable or increasing heritabilities. The pattern of twin correlations found here also indicate that there might be an influence of nonadditive genetic effects. If these effects were significant, they would contribute even more to a decreased DZA twin similarity⁸. Our data also indicate that within the range of practice studied here, these differences are based to a considerable degree on differences in genotypes. This conclusion does not diminish the importance of practice with feedback for the acquisition of skill. Even the least gifted of our twins attained levels of skill after practice that were superior to those achieved in initial trials by the most gifted. Nevertheless, the main findings of our study unequivocally show the important contribution of genotypic factors underlying individual differences in skill acquisition on the rotary pursuit task. □

Methods

Subjects. The participants were 64 pairs of identical twins reared apart (MZA) and 32 pairs of same-sex fraternal twins reared apart (DZA) who participated in the Minnesota study of twins reared apart between 1979 and 1994 (ref. 9). Of the MZAs, 42% were male (age, 44.2 years; s.d., 15.51), 58% female (age, 40.97; s.d., 12.39). Of the DZAs, 31% were male (age, 44.20; s.d., 13.52), 69% female (age, 43.73; s.d., 11.54).

Apparatus and procedures. Participants were tested individually during three successive morning sessions, of roughly 30 min each, on the pursuit rotor task. We used a pursuit rotor apparatus (Lafayette Instrument Co.) that rotated at a constant speed of 60 r.p.m. in a clockwise direction. A standard hinged stylus and standard instructions were used. A timing mechanism calibrated in 0.01-s units was used. The task was to keep the stylus tip in contact with the moving target using the preferred hand. Trial duration was 20 s, with a 10 s intertrial interval. Twenty-five trials were conducted per daily session. Each trial began with the stylus tip in contact with the target. Participants were given information feedback in units rounded to 0.1 sec, and were occasionally encouraged to 'keep trying as hard as possible'.

Data analysis. An efficient approach to estimating the significance of genetic and environmental (non-familial) effects in these data involved specifying and evaluating various biometric models¹⁰. This was accomplished by comparing the covariances of MZA and DZA twins. Maximum likelihood procedures were used to estimate genetic (H^2) and environmental (E^2) parameters of the various biometric models that have been specified¹¹. The maximum likelihood procedure also provides a χ^2 test of the fit of each particular model, as well as a χ^2 difference test for comparing the relative explanatory power of different models. Two models were examined here. One model assumed only non-shared environmental influences (in the absence of twins reared together, it is not possible to estimate shared familial components). A second model included genetic and environmental parameters. This combined model assumed that all genetic effects are additive, and that there is no assortative mating. Nonadditive genetic effects were not estimated owing to the difficulty of distinguishing additivity from nonadditivity reliably with small samples¹².

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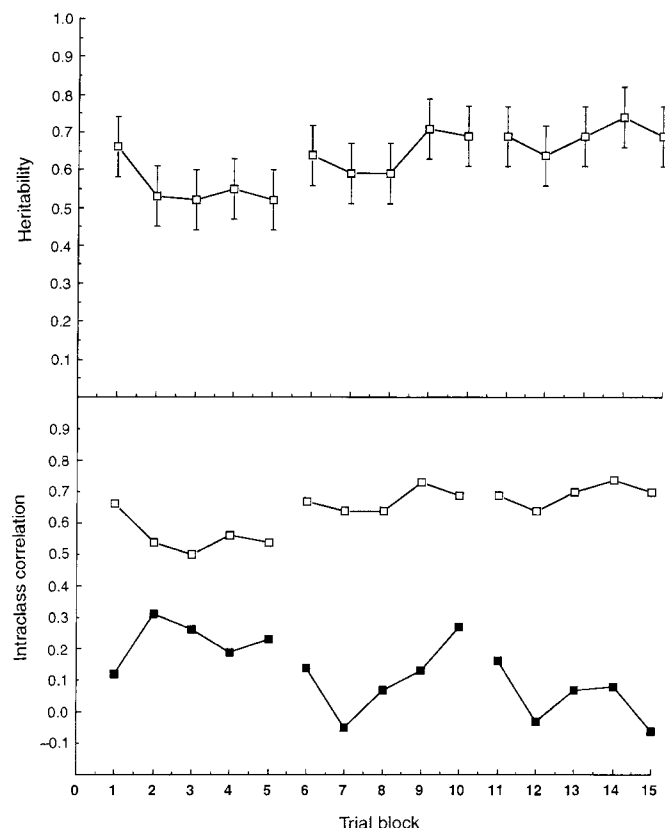


FIG. 2 Heritabilities estimated from model-fitting the twin data for each of 15 trial blocks (upper figure) ($\pm$ s.e.) and intraclass correlations for MZA and DZA twins for percentage of time-on-target for the rotary pursuit task (lower figure) MZA twins, open squares; DZA twins, filled squares.

TABLE 1 Rotary pursuit task performance

Day	Measure	MZA (N = 64)	DZA (N = 32)
1	Slope	0.56 (0.37–0.71)	0.24 (–0.11–0.54)
2		0.69 (0.54–0.80)	0.17 (–0.18–0.48)
3		0.72 (0.58–0.82)	0.11 (–0.24–0.43)
1–2	Reminiscence	0.19 (–0.06–0.41)	0.22 (–0.13–0.52)
2–3		0.55 (0.35–0.70)	0.51 (0.20–0.72)

Intraclass correlations and 95% confidence intervals for slopes and reminiscence measures on the rotary pursuit task for monozygotic and dizygotic twins that were reared apart. Because day 3 of testing was added after the study began, a smaller number of MZA twin pairs (58) participated on Day 3.

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Orthogonal motion after-effect illusion predicted by a model of cortical motion processing

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THE motion after-effect occurs after prolonged viewing of motion; a subsequent stationary scene is perceived as moving in the opposite direction^{1,2}. This illusion is thought to arise because motion is represented by the differential activities of populations of cortical neurons tuned to opposite directions; fatigue in one population leads to an imbalance that favours the opposite direction once the stimulus ceases³. Following adaptation to multiple directions of motion, the after-effect is unidirectional^{4–6}, indicating that motion signals are integrated across all directions. Yet humans can perceive several directions of motion simultaneously^{7–10}. The question therefore arises as to how the visual system can perform both sharp segregation and global integration of motion signals. Here we show in computer simulations that this can occur if excitatory interactions between different directions are sharply tuned while inhibitory interactions are broadly tuned. Our model predicts that adaptation to simultaneous motion in opposite directions will lead to an orthogonal motion after-effect. This prediction was confirmed in psychophysical experiments. Thus, broadly tuned inhibitory interactions are likely to be important in the integration and segregation of motion signals. These interactions may occur in the cortical area MT, which contains motion-sensitive neurons with properties similar to those required by our model^{11–14}.

Two populations of dots moving in different directions are readily perceived as two transparently moving sheets¹⁵. After adaptation to such 'transparent motion', observers never perceive a transparent motion after-effect (MAE), but rather a unidirectional MAE, opposite to the vector sum of the adaptation directions^{4–6}. This non-transparent MAE cannot be explained by the standard ratio model proposed by Sutherland¹⁶, in which motion is perceived as an imbalance of activities in a pair of oppositely tuned motion detectors. Following adaptation, one of the members of such a pair will respond below baseline. Through opponency between the two members this results in temporary activation of the previously inactive member, thus causing the percept of an MAE. The non-transparent MAE suggests that motion information is integrated across all directions, and not just across opponent pairs of directions. The distribution-shift model⁴ accounts for this MAE by a global shift in activities across a

population of motion-encoding neurons, due to adaptation. However, it does not explain why multiple motion vectors are perceived during the adaptation phase, while only a single motion vector is perceived during the test phase. To address this paradox, we added broadly tuned inhibition between motion directions to the distribution-shift model. The expanded model is aimed at understanding motion integration and segregation, in particular how transparent motion can cause a non-transparent MAE¹⁷.

The model has two stages, based on the physiology of motion perception in primates. A motion-detection stage, corresponding to directionally selective cells in area V1¹⁸, feeds into a global motion integration stage, corresponding to area MT. Motion detectors have a bandwidth of about 70°, which is typical of V1 neurons¹¹. Motion detectors excite second-stage units of similar preferred direction with a directional divergence from stage 1 to stage 2 of 30°. As a result, stage 2 units have a bandwidth of about 90°, similar to the bandwidth of MT neurons¹¹. Moreover, inhibitory inputs to stage 2 are driven by the output from stage 1 with a bandwidth of 180°, thus normalizing responses at stage 2^{19,20}. These interactions between different motion directions are similar to those observed in area MT¹². Perceived motion corresponds to supra-threshold peak(s) in the distribution of motion-sensitive cells in the second stage, analogous to the findings in area MT of macaques¹³ and humans¹⁴. The strength of the motion percept is related to the response amplitude, while the width of the response distribution relates to the sharpness of the perceived motion direction. The MAE can be explained by adaptation of motion detectors when they are activated. This has been implemented in our model through weights for the output of stage 1 that decay when motion detectors are activated, and recover after stimulus offset²¹. The time constant for this process is about 10 seconds (refs 22, 23). During the test phase (following adaptation) all stage 1 detectors are weakly active, especially when a dynamic test stimulus is used that consists of incoherent motion. Adapted units are temporarily suppressed, which causes disinhibition of oppositely tuned units at stage 2, thus causing an MAE. The model accounts for segregation of transparently moving patterns during adaptation, as well as global integration during the test phase as previously shown^{4–6}. The broad tuning of inhibition causes a broad MAE, agreeing with the finding that the MAE is indistinguishable from directionally biased noise².

The model simulations (Fig. 1a) show that transparent motion induces a unidirectional MAE. Adaptation to opposite directions causes no MAE parallel to the adaptation directions, in agreement with psychophysical results⁴. The model, however, suggests that adaptation to opposite motion directions can cause an MAE, provided that disinhibition is sufficiently strong and broad. In particular, motion during adaptation causes broadly tuned disinhibition in the test phase, leading to maximal combined disinhibition in directions orthogonal to the adaptation vectors. The model thus predicts an orthogonal MAE for oppositely directed motion. Simulation results show that the same model can account for both a vector average MAE (Fig. 1a) and an orthogonal MAE (Fig. 1b). The orthogonal MAE is a surprising prediction that runs counter to recent research showing vector addition in MAEs^{4–6}.

We tested this prediction in a psychophysical experiment. We adapted subjects to motion, and asked them to report the motion they perceived when viewing randomly moving dots. During the adaptation phase two populations of dots moving in opposite directions were displayed on the screen (Fig. 2a). There were four adaptation conditions: the two populations could move along the vertical, the horizontal or the two diagonals. During the test phase displacements for all dots were drawn from a distribution spanning all 360 degrees, thus creating incoherent motion (Fig. 2b). The motion displacements in the test phase were globally balanced to ensure that no directional biases were present. Subjects had to report whether they saw motion during the test phase, and if so, to indicate the axis along which they saw motion. The axis was not directed; for example, subjects could report motion along the vertical, but no distinction was made between up

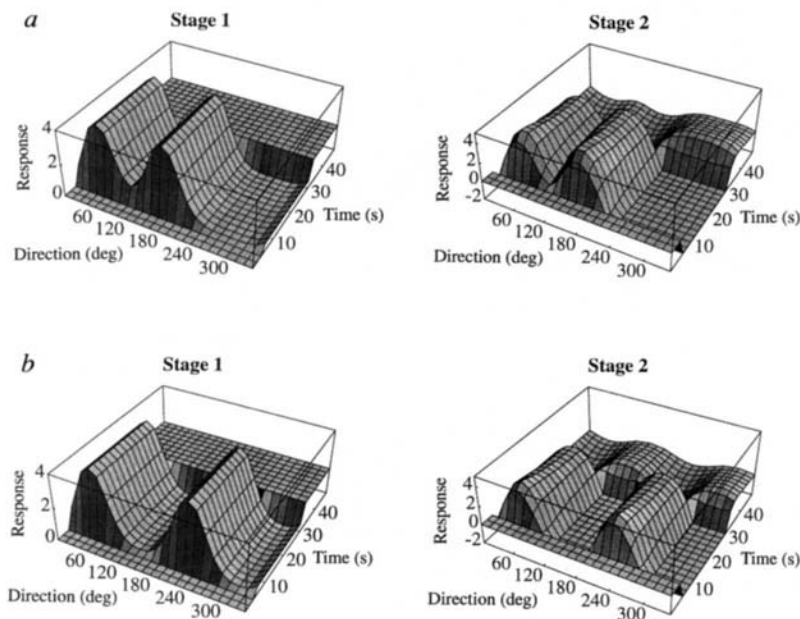


FIG. 1 Model simulations. *a*, Vector-average MAE: during the adaptation phase ($0 < t < 30$) two directions of motion (45° and 165°) are present, causing two peaks of activity at stage 1. The weights associated with the active units decay. Stage 2 units pool stage 1 activities. During the test phase ($t > 30$) all directions of motion are present, causing low, uniform activity at stage 1. Previously active detectors have adapted (their weights have decayed), so they send less inhibition to stage 2. Broad tuning of inhibition makes the two inhibitions overlap substantially, and thus the summed inhibition that falls away is unimodal, not bimodal. The single peak, corresponding to a unitary MAE, lies between the two directions opposite to the adaptation directions. Therefore, the stage 2 response is maximal in the direction opposite to the vector average of the adaptation directions. *b*, Orthogonal MAE: the adaptation stimulus contains opposite directions of motion (45° and 225°). The activities at both stages are slightly shifted compared to the vector-average MAE. As in *a*, during the test phase previously active units at stage 1 send less inhibition to stage 2. As the directions are opposite to each other, maximal disinhibition occurs at the two directions orthogonal to the adaptation directions, corresponding to an orthogonal MAE.

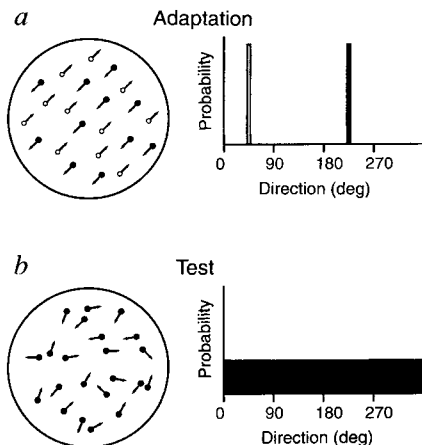
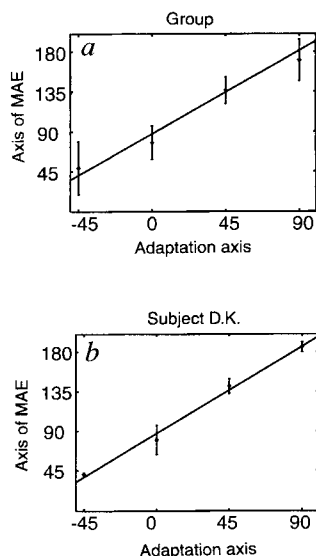


FIG. 2 Experimental stimuli. Left, cartoon of individual dot motion. Filled and empty circles are used to indicate the two different dot populations used. In the experiment all dots were black. Right, distribution of displacement vectors. *a*, Adaptation; *b*, test.



or down. Four naive subjects participated in the experiment. Subjects always reported that they saw motion during the test phase, that is, they were experiencing an MAE. Analysis of variance shows this effect to be highly significant ($P < 0.0001$). In particular, subjects reported seeing motion in the axis perpendicular to the adaptation stimulus for each condition (Fig. 3). A linear regression of reported axis versus adaptation axis has a slope of 0.9, and shows that the offset between adaptation axis and reported axis is 87.4° ($r^2 = 84\%$, $P < 0.001$). A *t*-test shows that the regression line is not significantly different from a line with unity slope and 90° offset ($P > 0.5$), thus confirming that the MAE was orthogonal to the inducing motion. This effect is very robust, which is reflected in the high level of confidence in their matches subjects reported after the experiment. Subjects clearly saw motion along the orthogonal axis, but when asked along which direction, they gave several answers: one of the two orthogonal directions, or both, or the percept alternated. This phenomenology is similar to transparent motion at low signal-to-noise ratios²⁴.

In our experiment we used a dynamic, rather than static, test display. Subjects perceive a dynamic MAE as real motion², which makes it easier to match the perceived axis of motion. Unlike static test patterns, dynamic test patterns do not create the percept of a coherent and stationary pattern, which might interfere with the orthogonal MAE. The use of a static test pattern is probably the main reason why others reported no MAE for opposite adaptation directions⁴⁻⁶. Indeed, repeating our experiment with a static test pattern caused no MAE. There is a long history of explaining MAEs in terms of pursuit eye movements. We think that eye movements during adaptation do not play a role in our experiments because they would not induce an orthogonal MAE. To support this we performed a control experiment in which we placed the fixation mark well outside the aperture through which subjects saw the stimulus. As a consequence, subjects could not

FIG. 3 Experimental results. *a*, Group summary. The graph shows the mean data across subjects, error bars give the standard deviation across subjects. Subjects always reported perceiving motion when viewing the test stimulus. The reported axis of motion during the test phase was orthogonal to the axis of motion of the adaptation stimuli. The data is fitted with a least-squares regression line ($y = 0.9x + 87.4$, $r^2 = 84\%$). Note that all angles are equal modulo 180 degrees; thus the adaptation axis value of -45° is equivalent to 135° . *b*, Single subject. The graph shows representative data for one subject, error bars give the standard deviation across trials. The data is fitted with a least-squares regression line ($y = 1.1x + 87.2$, $r^2 = 97\%$).

track the moving dots. They still reported seeing an orthogonal MAE (analysis of variance, $P < 0.001$; regression, $y = x + 87.6$, $r^2 = 97\%$), suggesting that eye movements play no role in the orthogonal MAE.

Our results show that the aftereffect from oppositely and transparently moving patterns does not always cancel. The proposed model accounts for integration and segregation of real motion during adaptation, and for different MAEs during the test phase. The orthogonal MAE supports the main assumptions underlying the model: (1) all motion directions interact in the integration of motion signals, (2) narrowly tuned excitation provides the excitatory interaction required for global integration^{25,26}, and (3) broadly tuned inhibition determines whether different directions are integrated or remain segregated. Other models of motion perception have focused on computational principles the visual system might use to process transparent motion⁸. Those models predict no MAE in our experiments because they do not include inhibition as a key factor in global motion processing. Studies of area MT have shown the importance of inhibition in the shaping of neural responses^{12,27}. Our results suggest that these inhibitory interactions play a major role in defining how motion signals are integrated and segregated. □

Methods

Model simulations. By simulating directional interactions, and no spatial interactions, we collapsed space into one location. Although locally only one direction of motion can be perceived (balanced motion appears as flicker²⁸, transparent motion segregates in depth²⁹), we model global motion phenomena, where multiple directions are perceptible. Thus collapsing space is permissible. We simulated the model as a system of differential equations capturing the dynamic properties of adaptation¹⁷. During adaptation responses are stronger and more narrowly distributed than during the test phase. Thus, MAEs are less compelling than real motion, and the direction is less sharply defined. The model 'perceived' an MAE when the simulated neural response at stage 2 in the test phase exceeded stage 2 responses to a stimulus with 10% signal to noise ratio, which is the psychometric and neurometric threshold^{2,13}. When two separate peaks exceed threshold, transparent motion is perceived. As described in the text, inhibition normalizes activities at stage 2, thus comparing activities across all directions. Alternatively, this comparison could be made explicit, through division or subtraction across all stage 2 units. This is equivalent to broad inhibition in our model. The model uses gaussian tuning curves. Stage 1 bandwidths mirror tuning of direction selective cells in V1 (70°). Stage 2 bandwidths are the sum of motion-detector bandwidths at stage 1 and excitatory divergence bandwidths between stages 1 and 2. The excitatory divergence bandwidth was adjusted to obtain stage 2 bandwidths that mirror those in MT (90°). Stage 2 bandwidths determine the minimal angle between directions for which bidirectional motion is segregated. We parametrically varied the bandwidths by up to 60° from the physiologically defined value. The model reproduced a vector-average MAE and an orthogonal MAE over a wide range of bandwidth combinations, provided that the stage 2 bandwidth did not exceed the difference between the adaptation directions, in which case the model failed to segregate transparent motion during adaptation. The bandwidth of inhibition between stages 1 and 2 is broad, to allow interactions between different directions. We tried bandwidths between 60° and 240°. The model performed well when the inhibitory bandwidth exceeded 120°. Below that bandwidth the orthogonal MAE stays below threshold.

Experimental procedure. Subjects were adapted for 30 s to transparent motion consisting of two opposite directions, after which they saw a test stimulus consisting of incoherent motion for 3 s. During the test phase the two ends of an imaginary line going through the centre of the aperture were displayed outside the aperture. The subjects' task was to indicate whether motion was perceived during the test, and if so to adjust the orientation of the line to the perceived axis of motion. Subjects could adjust the orientation of the axis in steps of 1°. The test was followed by 15 s of top-up adaptation, after which another test phase occurred. Testing was continued until the subject was convinced of the accuracy of the response. Trials were separated by at least 30 s during which subjects viewed a stationary, textured screen. Motion stimuli were random dot patterns consisting of black dots (4-arcmin diameter) on a white background. Each population of dots consisted of 250 dots, yielding a total dot density of 10 dots per degree². Dots were displaced by 7 arcmin between screen refreshes, which occurred at a rate of 72 Hz, resulting in a speed of 8 deg s⁻¹. Random dot patterns were displayed in a circular aperture of 7° diameter, eliminating directional biases due to screen geometry. Subjects fixated a central fixation marker during both the adaptation and test phases. Trials of the four adaptation conditions were randomly interleaved; each condition was repeated four times for each subject.

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Inflammatory pain hypersensitivity mediated by phenotypic switch in myelinated primary sensory neurons

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PAIN is normally evoked only by stimuli that are sufficiently intense to activate high-threshold A δ and C sensory fibres, which relay the signal to the spinal cord. Peripheral inflammation leads to profoundly increased pain sensitivity: noxious stimuli generate a greater response and stimuli that are normally innocuous elicit pain. Inflammation increases the sensitivity of the peripheral terminals of A δ and C fibres at the site of inflammation¹. It also increases the excitability of spinal cord neurons^{2,3}, which now amplify all sensory inputs including the normally innocuous tactile stimuli that are conveyed by low-threshold A β fibres. This central sensitization has been attributed to the enhanced activity of C fibres⁴, which increase the excitability of their postsynaptic targets by releasing glutamate and the neuropeptide substance P^{5–7}. Here we show that inflammation results in A β fibres also acquiring the capacity to increase the excitability of spinal cord neurons. This is due to a phenotypic switch in a subpopulation of these fibres so that they, like C-fibres, now express substance P. A β fibres thus appear to contribute to inflammatory hypersensitivity by switching their

phenotype to one resembling pain fibres, thereby enhancing synaptic transmission in the spinal cord and exaggerating the central response to innocuous stimuli.

Primary sensory neurons are highly specialized to transduce, transfer and transmit sensory information from the periphery to the central nervous system. The chemical diversity of the cells reflects their functional heterogeneity. The tachykinin substance P, which is involved in nociceptive central transmission and is present in about 25% of all primary sensory neurons, is normally expressed in about 45% of the high-threshold A δ and C-fibre sensory cells, coexpressed with the high-affinity nerve growth factor (NGF) receptor trkA⁸. There are C-fibres that do not express trk receptors or the neuropeptide and there are low-threshold cells with myelinated A β -fibres (~30% of total primary sensory neurons) that express trkB and trkC receptors but not substance P. The expression of substance P, like other neuropeptides, is not fixed, however. Depriving sensory neurons of contact with their targets by a peripheral axotomy results in a downregulation of substance P in C-fibres, rescued by supplemental NGF^{9,10} and increased expression in dorsal column myelinated fibres¹¹. But inflammation, which increases NGF levels in the periphery¹², results in the upregulation of preprotachykinin-A (PPT-A) messenger RNA¹³ and substance P¹⁴ in an NGF-dependent fashion.

We have investigated whether experimental inflammation alters the chemical phenotype of A β -fibres, and whether this changes their central actions. An acute, sterile, localized inflammation with oedema, erythema and increased sensitivity was produced, under anaesthesia, by intraplantar injection of turpentine oil. The force required to elicit a flexion withdrawal reflex from the inflamed tissue fell from >60 g to <10 g (Fig. 1a), and a light tactile stimulus to the inflamed paw elicited hindpaw elevation. The mechanical and thermal hypersensitivity was, as in other models^{12,15,16}, NGF dependent. Pretreatment with 5 μ g l anti-NGF serum¹² before the inflammation resulted in a mechanical

threshold of 20 ± 3.6 g and a thermal reaction time of 16 ± 1.5 s ($n = 3$, hotplate 50°C), significantly higher ($P < 0.001$) than the 7.2 ± 1.2 g threshold and thermal reaction time of 6.7 ± 1.2 s ($n = 3$) with control serum. Intermittent repeated low-intensity tactile stimulation of the dorsal surface of the inflamed hindpaw produced a progressive further reduction in the flexion reflex threshold tested in the same area as the conditioning stimuli (Fig. 1b). This is the phenomenon of progressive tactile hypersensitivity¹⁷, which indicates that low-intensity stimuli after inflammation begin to generate incremental sensitization, something they never normally do. This might be due to a reduction in threshold of nociceptors in the periphery, or to a change in the response evoked by low-threshold fibres in a spinal cord, as a consequence of a change in their transmitter content. We have investigated the latter possibility.

The inflammation resulted in an elevation in substance P content in the dorsal root ganglia innervating the inflamed hindpaw (Fig. 1c). The substantial increase in the number of dorsal root ganglion cells expressing preprotachykinin A mRNA (Fig. 1d) implies new expression in cells normally negative for substance P rather than mere upregulation in cells normally positive for substance P. To test whether this new expression occurred in A-fibres, we measured normally and following inflammation the overlap between dorsal root ganglion (DRG) cells expressing substance P and cells that possess myelinated axons. The sciatic nerve was injected with the B fragment of cholera toxin (CB). CB binds to GM1 ganglioside found only on A-fibres¹⁸, and substance P expression was detected immunohistochemically (Fig. 2). In control animals $13.9 \pm 1.8\%$ of substance P immunoreactive DRG profiles were double labelled for CB, most of which are likely to possess small myelinated axons (A δ fibres). After inflammation (48 h) this increased to $21.8 \pm 2.0\%$ ($n = 4$, $P = 0.02$, Mann-Whitney), and was associated with an increase in the area of the substance P-positive cell profiles from $512 \pm 12 \mu\text{m}^2$ ($n = 235$) to $582 \pm 10 \mu\text{m}^2$ ($n = 491$) ($P < 0.0001$,

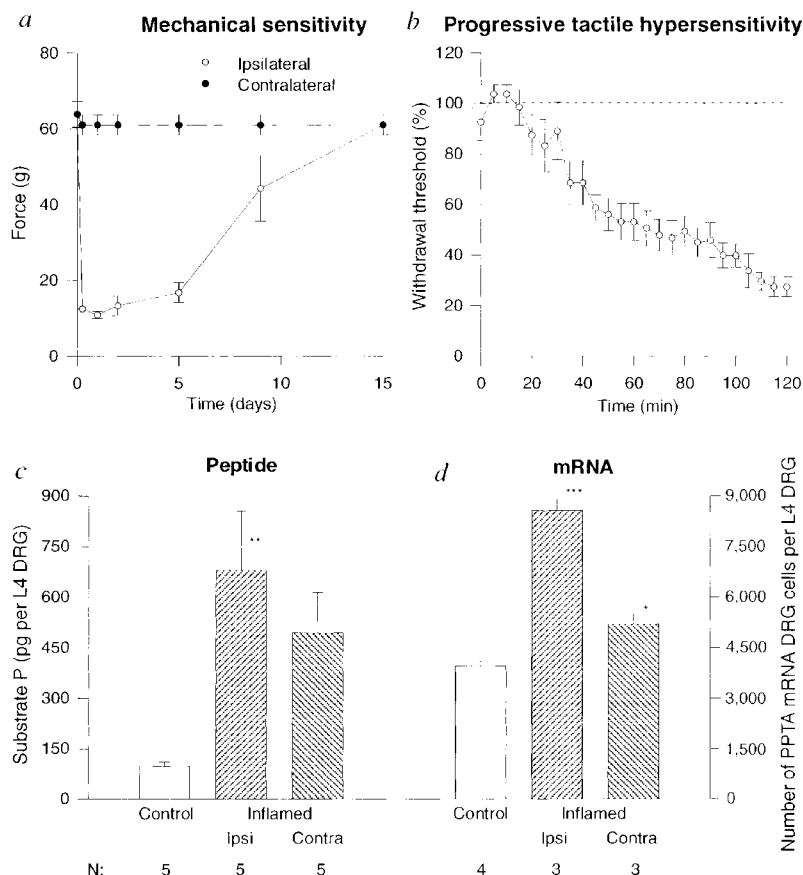


FIG. 1 a, Acute inflammation reduces the mechanical threshold for eliciting a flexion withdrawal reflex ($n = 5$). b, Repeated light tactile stimulation of an inflamed paw (48 h) generates a progressive further reduction in the threshold of the flexion reflex ($n = 5$). Threshold testing alone had no such effect ($n = 5$). Results are normalized with respect to the threshold values immediately preceding the tactile test stimuli. c, The amount of substance P in the L4 dorsal root ganglion increases significantly 48 h after inflammation. d, The number of DRG cells expressing preprotachykinin A (PPT-A) mRNA increases with inflammation (48 h). All results are expressed as mean $\pm$ s.e.m. and significant differences calculated by Welch's *t*-test (* $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$).

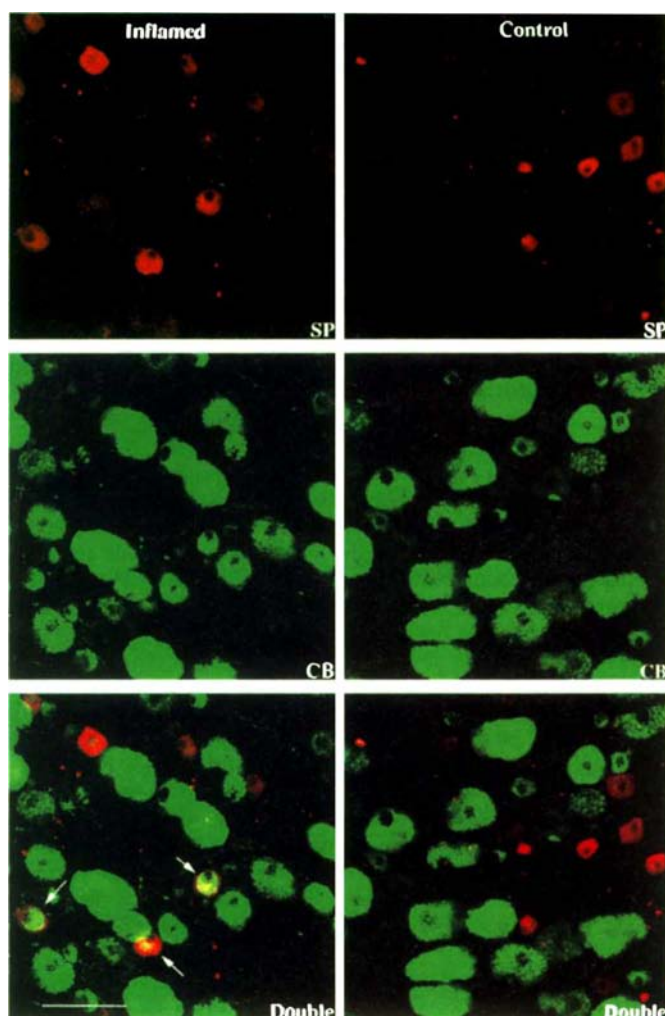


FIG. 2 Confocal photomicrographs showing images constructed from 16 optical sections through a 50- μ m section of a dorsal root ganglion from a control and inflamed animal labelled for substance P, the B fragment of cholera toxin (CB; a marker of myelinated axons) and both together. After inflammation many cell bodies positive for cholera toxin express substance P (arrows), whereas in the control, fewer cells are double labelled. Scale bar, 100 μ m. The very faint label is nonspecific background.

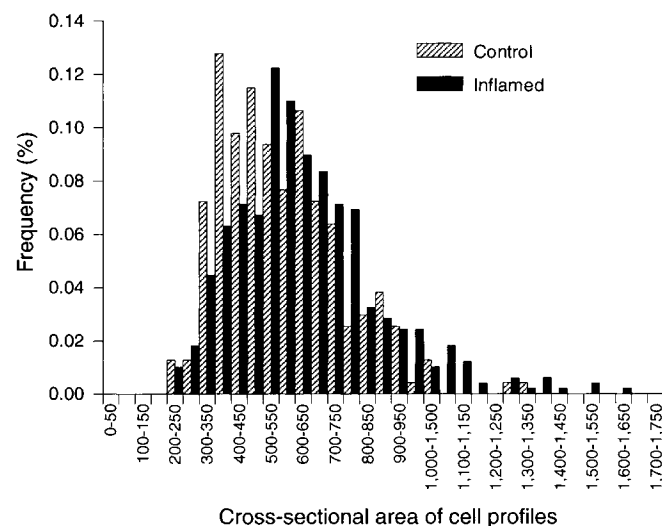


FIG. 4 Confocal photomicrographs of transverse 100- μ m-thick sections of L4 dorsal roots stained for substance P immunofluorescence. Control shows substance P immunofluorescence only in the small fibres interposed between the large myelinated axons. After inflammation, substance P immunofluorescence increases in both large myelinated fibres (arrows) and in the small C and A δ fibres. In order to visualize the myelinated fibres, the top two confocal images generated from four optical sections through the tissue are superimposed on a transmission light image. The bottom image is not superimposed, and illustrates that the label in the myelinated fibres is distinct from that in the unmyelinated fibre bundles. Counts of immunoreactive myelinated fibres could be accurately resolved only in those fibres with a diameter $>6\mu$ m ($\sim 70\%$ of total myelinated fibres). Scale bar, 50 μ m.

FIG. 3 A size-frequency histogram illustrating the distribution of the cross-sectional areas of the profiles of substance P-immunoreactive dorsal root ganglion cells pooled from 4 control and inflamed animals ($n = 235$ control, 491 inflamed). Inflammation resulted in an overall shift in the distribution of the size of the profiles to the right due both to an increase in size of small cells and the appearance of a group of large ($>950\mu\text{m}^2$) substance P-labelled profiles. The mean area in each inflamed animal matched with a control processed at the same time was always larger; 587 versus 542, 471 versus 433, 689 versus 529 and 593 versus 508 μm^2 .

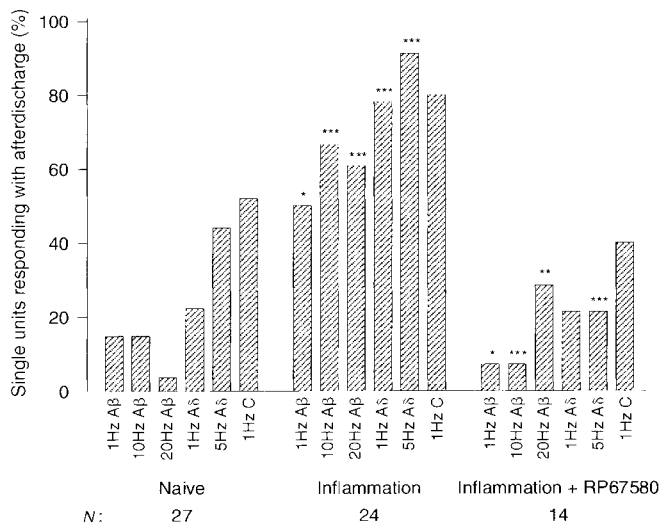


FIG. 5 The proportion of deep dorsal horn wide dynamic range cells showing action-potential afterdischarge following a 20-s train of stimuli to the sciatic nerve at current strengths that successively activate Aβ (100 μA, 50 μs), Aδ (500 μA, 50 μs) and C fibres (5 mA, 500 μs). In naive animals, afterdischarge is only evoked after high-frequency Aδ, or C-fibre strength stimuli. In inflamed animals (48 h) all test stimuli elicit afterdischarge, and Aβ 1 Hz stimuli now produces as much afterdischarge as C-fibres in the control situation. The administration of 1 mg per kg RP67580 to inflamed animals substantially reduced the capacity of all the test stimuli to produce afterdischarge. Recordings were made from 6 naive animals, 5 inflamed and 4 inflamed + RP67580. Differences calculated using the chi-square test.

Welch's *t*-test) (Fig. 3). Because the sciatic nerve and the L4 dorsal root ganglion contain many fibres/cells not innervating the inflamed tissue, these changes are underestimates. The area of CB-positive DRG cells, ($1,129 \pm 70 \mu\text{m}^2$, $n = 582$ control; $1,026 \pm 61 \mu\text{m}^2$, $n = 1,022$ inflamed, $P = 0.2$ pooled from 4 animals per group), and their the density of labelling (22.5 ± 4.6 profiles per $400 \mu\text{m}^2$ control and 21.0 ± 2.6 profiles per $400 \mu\text{m}^2$ inflamed, $n = 4$) did not alter, nor did CB conjugated to horseradish peroxidase (HRP) transganglionically label lamina II of the dorsal horn 48 h after the inflammation ($n = 2$), showing that inflammation did not lead to CB labelling of C-fibres. Direct evidence that substance P is expressed in Aβ fibres after inflammation was provided by counting the number of large ($>6 \mu\text{m}$) Aα and Aβ myelinated fibres in the L4 and L5 dorsal roots immunoreactive for substance P (Fig. 4). The total number of substance P-positive large myelinated axons/dorsal root increased threefold from 19 ± 3 in controls to 62 ± 7.6 in inflamed animals ($P = 0.002$, Welch's *t*-test, $n = 6$), which will underestimate the degree of change in fibres innervating the inflamed tissue. This change may reflect true new expression or an elevation from presently undetectable levels.

Given the shift in the phenotype of myelinated fibres innervating inflamed tissue to one resembling C-fibres, does the synaptic drive generated by A-fibres also alter? This was examined by looking at action-potential afterdischarge elicited in deep dorsal horn wide dynamic neurons by brief stimulation of A or C-fibres (Fig. 5). In non-inflamed animals the incidence of afterdischarge to a 20 s Aβ (1, 10 to 20 Hz) or an Aδ (1 Hz) train was very low, only 5 Hz Aδ and 1 Hz C-fibre trains elicited afterdischarges in a large proportion of cells (Fig. 4). After inflammation a significant increase in the proportion of cells showing afterdischarge to Aβ trains and a further increase in response to Aδ and C inputs occurred. This was completely prevented by systemic administration of the NK₁ receptor antagonist RP67580, indicating that the increased afterdischarge is the consequence of substance P release, which is most likely to be of primary afferent origin, although substance P-expressing dorsal horn neurons¹⁹ may contribute. RP67580 (1 mg kg⁻¹, intraperitoneally (i.p.))

also significantly attenuated the degree of progressive hypersensitivity of the flexion reflex elicited by tactile stimulation of the inflamed hindpaw (Fig. 1b). The extent of the decrease in threshold at 110 min produced by intermittent light stroking of the inflamed skin was $75 \pm 6\%$ in inflamed animals without RP67580 and $42 \pm 8\%$ in its presence ($n = 5$, $P < 0.01$). The RP67580 had no effect on basal sensitivity before the tactile stimuli in the inflamed or control animals, and its inactive enantiomer RP68651 had no detectable effect either on the development of progressive tactile hypersensitivity or on basal sensitivity ($n = 5$). Neither drug affected the animals' general behaviour, alertness or locomotion.

Primary sensory neurons signal the onset, intensity, duration and location of peripheral stimuli to spinal cord neurons by fast glutamate-mediated synaptic currents. C-fibre afferents also, by virtue of the slow synaptic currents they evoke through *N*-methyl-D-aspartate (NMDA) and tachykinin receptors^{20,21}, modulate membrane excitability of dorsal horn neurons. The slow synaptic currents can summate temporally and spatially and raise internal calcium concentration ($[\text{Ca}^{2+}]$) through Ca^{2+} inflow and release of intracellular stores²². This produces a protein kinase C-dependent phosphorylation of the NMDA receptor partially removing its voltage-dependent Mg^{2+} blockade²³, increasing glutamate sensitivity, and recruiting subthreshold inputs²⁴. An increase in the number of Aβ fibres expressing substance P after inflammation results in a qualitative change in function; they can now, like C-fibres, induce increase central excitability *in vivo* and evoke prolonged ventral root potentials *in vitro*²⁵. Aβ fibres terminate in lamina III of the dorsal horn²⁶, an area where NK₁ receptor-expressing cells are located²⁷, explaining how a presynaptic transmitter change may initiate new postsynaptic alterations in excitability, although volume transmission may contribute. Inflammatory pain is an expression of multiple forms of neuroplasticity: changes in transduction sensitivity in the periphery, altered excitability in the spinal cord and, as we now show, a phenotypic switch in primary sensory neurons. □

Methods

Inflammation and behavioural sensitivity. Inflammation was generated by an intraplantar injection in adult male Sprague-Dawley rats of 100 μl of 50% turpentine oil (in paraffin oil) under 4% halothane anaesthesia. Mechanical sensitivity of the flexor reflex was measured using monofilament nylon von Frey probes¹². Progressive tactile hypersensitivity was elicited by 8 light touches applied manually to the dorsal surface of the hindpaw every 4 s, at 4-min intervals and measured with monofilament probes applied to the same site¹⁷.

Substance P measurements. Substance P levels were extracted from fresh DRGs and measured by an inhibition enzyme-linked immunosorbent assay (ELISA)¹². *In situ* hybridization was performed using an alkaline phosphatase-linked 30-mer oligodeoxynucleotide probe to PPT-A using hybridization, visualization and physical disector counting techniques as detailed before¹³.

Immunohistochemistry. Double label in the DRG: 1 μl of cholera toxin B subunit (CB) conjugated to fluorescein isothiocyanate (List, 5% in dH₂O) was injected into the left sciatic nerve of inflamed (24 h, $n = 4$) and naive rats ($n = 4$) under halothane anaesthesia. After 48 h the L4 and L5 dorsal root ganglia were removed after perfusion with 2% paraformaldehyde and 0.2% picric acid in 0.1 M phosphate. Frozen sections (50 μm) were cut and stained free floating with a polyclonal antiserum to substance P (1:8,000; Affinity) and visualized with a goat anti-rabbit IgG conjugated to tetramethyl rhodamine isothiocyanate (TRITC) (1:200; Vector). Images were analysed using a Leica laser-scanning confocal microscope (TCS4D). Sixteen optical sections ($400 \mu\text{m}^2$) were collected from each tissue section and reconstructed as a two-dimensional projection. TRITC stimulating wavelength 568 nm, fluorescein isothiocyanate (FITC) 488 nm. Profile counts were obtained from 5–10 non-adjacent sections per ganglion. Counts were made blind by two observers. Area measurements were only made from nucleated profiles. Substance P label in dorsal roots: the L4 and L5 dorsal roots from inflamed ($n = 6$) and control naive ($n = 6$) animals were removed and fixed overnight at 4 °C. Transverse frozen sections (100 μm) were cut and processed for immunocytochemistry for substance P as above. Four optical sections ($25 \mu\text{m}^2$) were collected from each tissue section and reconstructed as a two-dimensional projection.

Electrophysiology. Experiments were performed in decerebrate-spinal rats with the L4 segment exposed by a laminectomy. Dorsal horn recordings were made with gold/platinum-plated tungsten microelectrodes and the sciatic nerve stimulated with a constant current stimulator through bipolar silver electrodes²⁸.

Single spikes with a signal : noise ratio of $>20:1$ were recorded and spike shape monitored with an analogue-delay line. Afterdischarge was defined as the presence of spike activity above mean ± 2 s.d.s of background activity counted over 20 s.

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Dendritic cells capable of stimulating T cells in germinal centres

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THE B cells within the germinal centres of lymphoid organs undergo affinity maturation of their antigen receptors, a critical event for antibody memory^{1–3}. Follicular dendritic cells within the germinal centres retain immune complexes^{4,6} that select the developing B cells for which they have a higher affinity⁷. We have now identified a subset of CD4⁺CD11c⁺CD3[−] dendritic cells in the germinal centres. These are strong antigen-presenting cells for T cells, but do not co-stimulate CD40-activated B cells. These dendritic cells probably stimulate germinal centre T cells and aid the complicated processes that are required for the generation of memory B cells.

This study stemmed from an intriguing histological observation that human tonsillar germinal centres (GCs) contain more CD4⁺ cells than CD3⁺ cells. As both epidermal Langerhans cells and blood dendritic cells were reported to express CD4 (ref. 8), we investigated whether GCs contained CD4⁺ dendritic cells (DCs).

Double immunohistological staining with anti-CD4 and anti-CD3 revealed a population of large dendritic CD4⁺CD3[−] cells within GCs, which were found in close association with GC T cells (Fig. 1a). They expressed CD11c, an antigen highly expressed on dendritic cells⁸ (Fig. 1b), but not the follicular dendritic cell (FDC) specific antigen that is recognized by monoclonal antibodies KiM4 (ref. 9), DRC-1 (ref. 10) and 7D6 (ref. 11) and has recently been identified as the CR2/CD21 long isoform¹² (Fig. 1c). In contrast to the low number and polarized localization of Tingible body macrophages in the GC basal light zones and dark zones (Fig. 1h), CD4⁺CD11c⁺ GCDCs were evenly distributed in both the dark and light zones (Fig. 1d). They represent 0.5–1% of total GC cells in human tonsils, spleens (Fig. 1e) and lymph nodes (Fig. 1f). Unlike interdigitating dendritic cells from the T-cell zones that were CD40^{high}, GCDCs were CD40^{low} (Fig. 1g). They did not express CD1a (not shown), a marker of epithelial Langerhans cells¹³.

The CD4⁺CD11c⁺CD3[−] phenotype of this dendritic cell population allowed the development of a multistep purification method including: (1) centrifugation through a 50% Percoll gradient; (2) magnetic bead depletion of B cells (CD19⁺CD20⁺), T cells (CD3⁺), monocytes (CD14⁺) and natural killer (NK) cells (CD56⁺); (3) further sorting for CD4⁺CD11c⁺ cells that are negative for CD20, CD3, CD14, CD1a (Langerhans cells), CD34 and CD16. Tingible body macrophages that displayed high autofluorescence were depleted by fluorescence gating. Interdigitating DCs isolated by sorting CD40^{high} tonsillar cells that were negative for lineage markers were found to express less CD4 (P. Björck and Y.-J.L., manuscript in preparation) and were not collected. Consistent with the dendritic morphology and phenotype of GCDCs *in situ*, the sorted cells displayed an eccentric nucleus, fine dendrites (Fig. 2a) and no surface markers specific for B cells, T cells, monocytes (Fig. 3), NKs (CD16 and CD56) or FDCs (KiM4/DRC-1/7D6/CD21 long isoform; results not shown). Although both germinal centre and FDCs express immune complexes, CD11b, CD32, CD35, CD44 and CD54, 13 other markers were differentially expressed on the two cell types (Fig. 3; Table 1)^{9–11,14,15}. In addition, GCDCs either freshly isolated or after overnight culture are smaller than FDCs and do not contain multiple big round nuclei (Fig. 2a, b, g–i, k). Isolated GCDCs did not have either nonspecific esterase (NSE) activity (Fig. 2c, d) or high levels of surface CD71 (Fig. 3), which are both markers for Tingible body macrophages¹⁶ (Table 1). Unlike CD40^{high} interdigitating DCs (Table 1) and CD1a⁺ Langerhans cells, sorted GCDCs were CD40^{low} (Fig. 3), CD1a[−] (not shown) and had no Birbeck granules when examined by electron microscopy (Fig. 2k). In addition, cultured GCDCs projected long dendrites and some aggregated into large clumps (Fig. 2e, f), a culture morphology similar to that of the DCs generated *in vitro*^{13,17–19}. Furthermore, cultured GCDCs strongly expressed major histocompatibility complex (MHC) class II antigens (Fig. 2g, h) and had all the ultrastructural characteristics of mature DCs (Fig. 2j, k)^{8,13}.

The sorted cells displayed similar characteristics to blood CD4⁺CD11c⁺ DCs, namely: (1) they had similar morphology; (2) their surface phenotype was similar (Fig. 3); and (3) all underwent spontaneous maturation *in vitro* by increasing dendrites and upregulating co-stimulatory molecules⁵. This indicates that blood CD4⁺CD11c⁺ DCs may be the precursors of GCDCs. Coexpression of Igκ and λ light chains suggested that immune complexes could be present, consistent with their expression of Fc receptors as well as complement receptors.

As the GCDCs were in close contact with GC T cells and GC B cells *in vivo*, their effects on T-cell and B-cell activation were analysed *in vitro*. Figure 4a shows that as few as 70 GCDCs could induce strong proliferation of allogeneic CD4⁺ T cells. This strong antigen-presenting-cell (APC) function may be explained by their ability rapidly to upregulate CD40, MHC class II and CD80 (B7.1) and CD86 (B7.2) in culture (Fig. 4b), as previously described for CD4⁺CD11c⁺ blood DCs⁸. In contrast, as many as 5,000 GC B

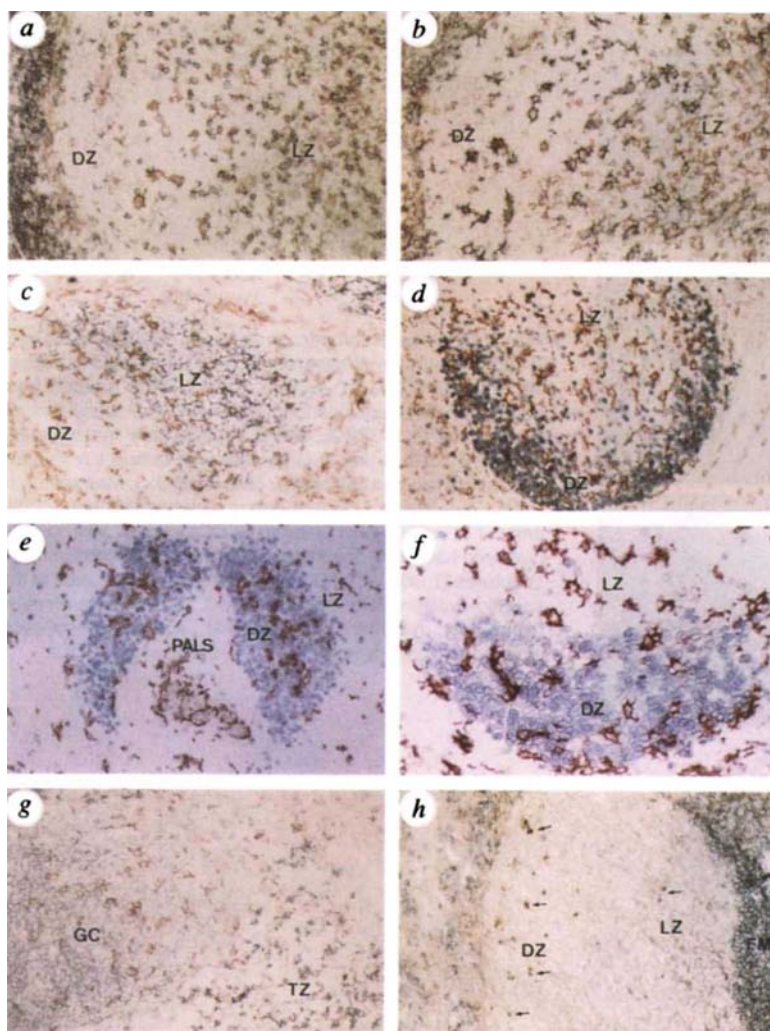


FIG. 1 Immunohistological characterization of $CD4^+CD11c^+$ dendritic cells in germinal centres. *a*, Anti-CD3 (blue) and anti-CD4 (red) double staining shows small $CD4^+CD3^+$ GC T cells (purple) and large $CD4^+CD3^-$ dendritic cells (red) within GC (magnification, $100\times$). *b*, Anti-CD4 (red) and anti-CD11c (blue) double staining shows that all large $CD4^+$ dendritic cells (purple) but not small $CD4^+$ T cells (red) express the dendritic cell marker CD11c (magnification, $100\times$). *c*, Anti-CD11c (red) and anti-FDC (blue, KiM4 or 7D6) staining shows that the $CD4^+CD11c^+$ GCDCs (red) do not express follicular dendritic cell (FDC)-specific antigens (blue) and FDCs do not express CD11c (red) (magnification, $100\times$). *d-f*, Anti-CD11c (red) and anti-Ki67 (blue nuclear) staining shows that $CD4^+CD11c^+$ GC dendritic cells are evenly distributed both in the GC dark zone (packed with Ki67 $^+$ centroblasts) and GC light zone of tonsils (*d*, $100\times$), spleen (*e*, $100\times$) and lymph node (*f*, $200\times$). The CD11c $^+$ cells within the periarteriolar lymphoid sheath (PALS) of spleen represent interdigitating dendritic cells (IDCs). *g*, Anti-CD11c (red) and anti-CD40 (blue) staining shows that IDCs in the T-cell zone (TZ) are strongly CD40 $^+$ (dark blue), $CD4^+CD11c^+$ GCDCs (red) are CD40 low ($100\times$). *h*, Anti-IgD staining shows the follicular mantle (blue) and the TUNEL technique³² shows apoptotic bodies within the Tingible body macrophages that are mainly located in the GC dark zone (magnification, $\times 100$).

TABLE 1 Comparison of phenotypes of germinal centre, follicular and interdigitating dendritic cells and Tingible body macrophages

Antigens	GCDC	FDC	IDC	TBM
CD2	+	—	—	—
CD4	+	—	Low	+
CD11c	+	—	Low	+
CD13	+	—	—	+
CD33	+	—	—	+
CD38	+	ND	ND	ND
CD45	+	—	+	+
CD21	Low	High	ND	Low
CD14	—	+	—	ND
DRC-1	—	+	—	—
KiM4	—	+	—	—
7D6	—	+	—	—
Immune complexes	+	+	+	ND
CD11b	+	+	ND	ND
CD23	Low	High and —	ND	ND
CD32	+	+	ND	ND
CD35	+	+	ND	ND
CD44	+	+	ND	ND
CD54	+	+	ND	ND
CD40	Low	+	High	+
CD80	—	ND	+	ND
CD83	—	ND	+	ND
CD86	—	ND	+	ND
CD71	Low	ND	—	+
NSE	—	—	—	+

GCDC, germinal centre dendritic cells; FDC, follicular dendritic cells; IDC, interdigitating dendritic cells; TBM, Tingible body macrophages; NSE, nonspecific esterase; ND, not determined.

cells isolated from the same tonsils failed to induce detectable T-cell proliferation. Unlike macrophages and monocyte-derived DCs^{19,20}, GCDCs have little ability to take up soluble antigen fluorescein isothiocyanate (FITC)-dextran (Fig. 4c) and to phagocytose FITC-latex beads, as examined by confocal microscopy and electron microscopy (not shown). In contrast to human FDCs or FDC-like cell lines^{11,21,22}, GCDCs did not significantly promote the proliferation of CD40-activated GC B cells *in vitro* (data not shown). The function of immune complexes on GCDCs with respect to B cells is not known, because our functional studies could not be performed in an antigen-specific human system.

We have demonstrated the presence in human GCs of two types of APCs: FDCs that promote the activation/selection of B cells, and DCs that strongly activate T cells. Although DCs are key cells for naive T-cell activation, memory T cell-DC conjugates have been found within the inflammatory synovial tissues, skin and mucosal membranes^{23,24}. The close physical contact of GCDCs with GC memory T cells *in situ* and their potent APC function suggest that they are important in maintaining GC reaction by sustaining the activation state of GC memory T cells²⁵. This finding does not exclude the possibility that GC B cells could present antigen to T cells²⁶. But as DCs in GC are more potent APCs compared with GC B cells, DCs may promote the T-B-cell interaction within germinal centres.

One of the most striking features of HIV pathology is the accumulation of HIV virus within germinal centres²⁷⁻²⁹. The expression of both immune complexes and CD4 on the surface of GCDCs suggests that they may contribute to this feature of HIV pathology. Furthermore, both *in vitro* and *in vivo* experiments indicate that replication of HIV-1 virus occurs during the

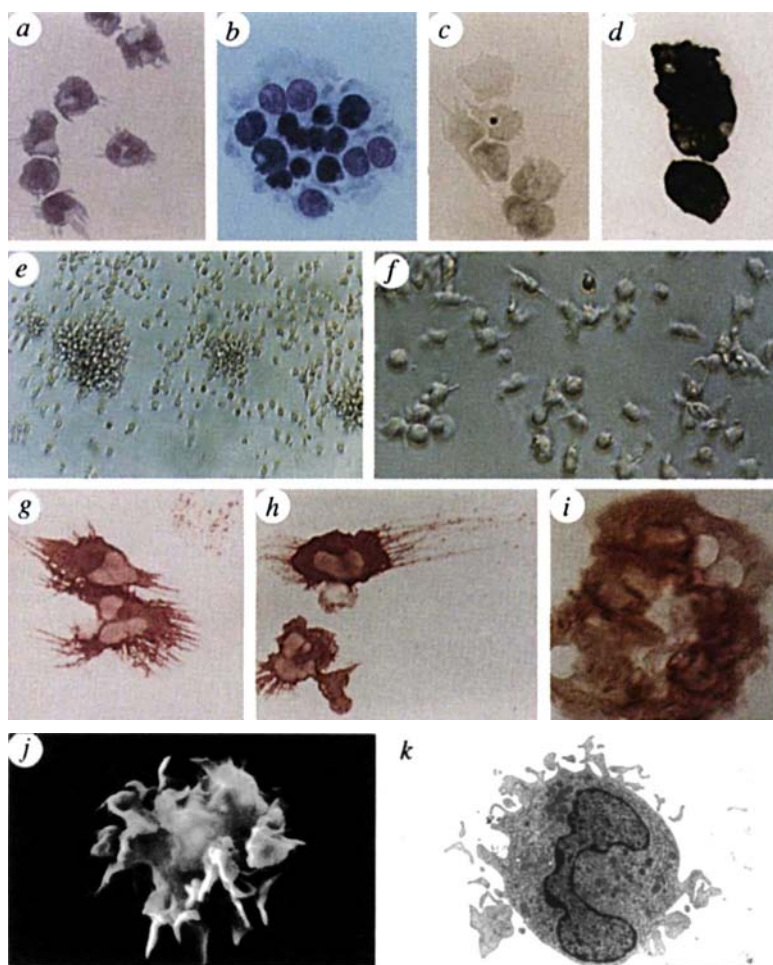


FIG. 2 Cytological comparison between GCDCs, FDCs and Tingible body macrophages from human tonsils. Giemsa staining of *a*, $CD4^+CD11c^+$ GCDCs, and *b*, FDCs isolated from human tonsils (magnification, $1,000\times$)¹¹. Nonspecific esterase staining of *c*, GCDCs, and *d*, Tingible body macrophages isolated by cell sorting of large cells with strong autofluorescence from low-density tonsillar cells ($1,000\times$). Morphology of GCDCs cultured for 16 h is shown in *e* at magnification $\times 200$, and in *f* at $\times 400$. *g*, *h*, Cultured GCDCs express MHC class II (red) and have long dendrites. Some cells bear iccosome-like structures ($1,000\times$). *i*, FDC characterized by expression of the specific antigen 7D6 (red, $1,000\times$). *j*, Scanning electron microscopy (magnification, $3,800\times$), and *k*, transmission electron microscopy (magnification, $1,860\times$) of GCDCs.

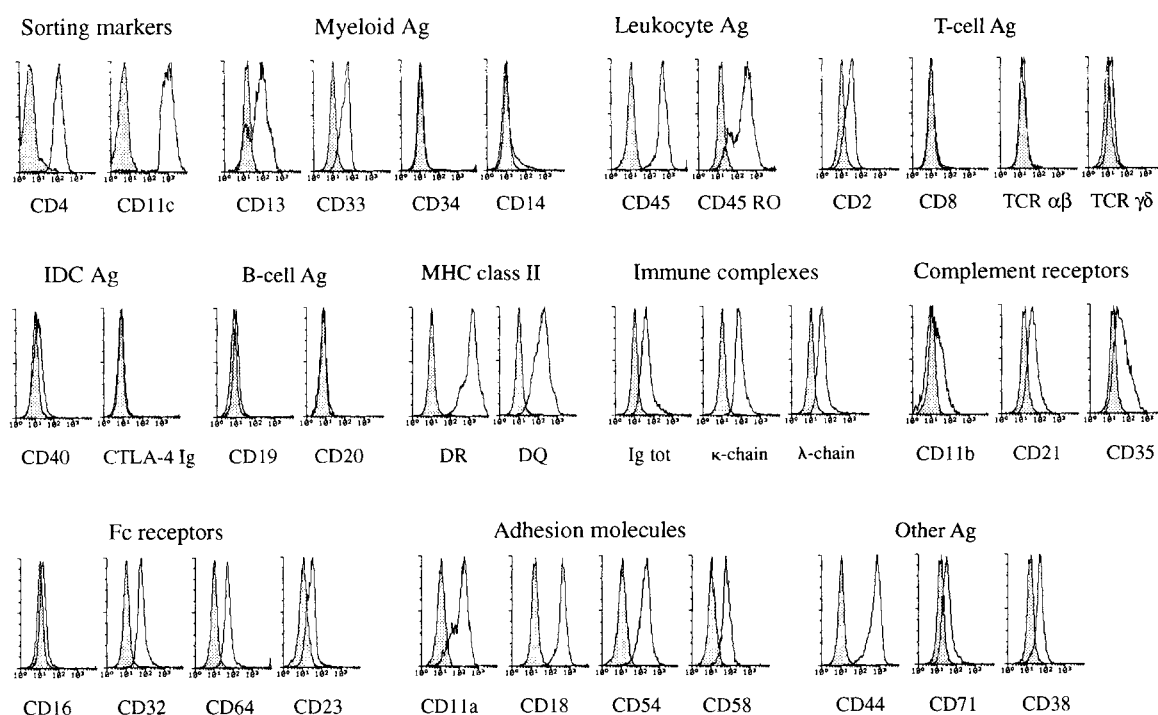


FIG. 3 Phenotypic analysis of isolated $CD4^+CD11c^+$ GCDCs. Expression of the molecules tested is shown in the white histograms and the isotype-matched controls are shaded. Ag, antigen.

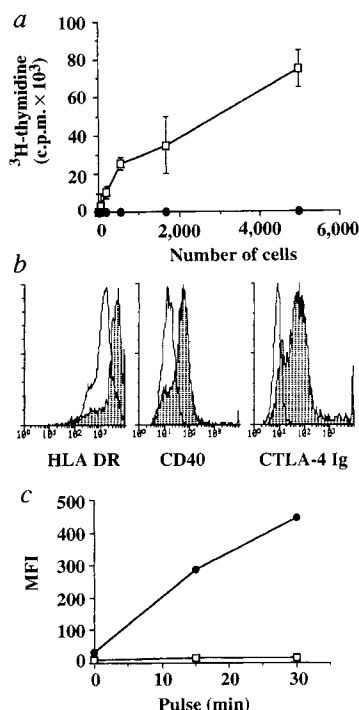


FIG. 4 a, GCDCs but not GC B cells induce strong CD4⁺ T-cell proliferation. ³H-thymidine uptake by T cells at the fifth day of co-culture with allogeneic GCDCs (squares) and with allogeneic GC B cells (circles). GCDCs and GC B cells were isolated from the same tonsils. b, Expression of MHC class II, CD40 and CTLA-4 ligands on freshly isolated GCDCs (white histograms) and after 16 h culture with RPMI 1640 containing 10% FCS (shaded). c, GCDCs have a poor ability to take up FITC-dextran. Methods are described in refs 18, 19. Time zero represents cells that were incubated with FITC-dextran for 30 min at 4 °C. After three washes, FITC-dextran uptake by GCDCs cells (squares) and monocyte-derived DCs (circles) at 37 °C were analysed using a FACScan flow cytometer. MFI, mean fluorescence intensity.

cognate CD4⁺ memory-T-cell activation by dendritic cells³⁰. The potent APC function of GCDCs and their close contact with CD4⁺ memory T cells suggests that GCs may provide a second important site for HIV viral replication, in addition to the mucosal surface³¹. □

Methods

Immunohistological characterization of CD4⁺CD11c⁺CD3⁺ GCDCs. Double staining was done using mouse IgG1 anti-CD3 (Immunotech), Kim4 (Behring), 7D6, Ki67 (Dako), CD40 (G 28.5; from E. Clark) and IgD (Dako), together with mouse IgG2a anti-CD4 (Innotest) and mouse IgG2b anti-CD11c (Becton-Dickinson). The binding of mouse IgG1 antibodies was revealed by sheep anti-mouse IgG1 (The Binding Site), followed by mouse anti-alkaline phosphatase-alkaline phosphatase complexes (Dako; APAAP technique). The binding of mouse IgG2a and IgG2b antibodies was revealed by sheep anti-mouse IgG2a-biotin and sheep anti-mouse IgG2b-biotin (The Binding Site), followed by ExtrAvidin peroxidase (Sigma). Alkaline phosphatase activity was revealed using Fast blue substrate; peroxidase activity was demonstrated by 3-amino-ethylcarbazole. Apoptotic bodies were detected using an *in situ* apoptosis detection kit (Oncor, Gaithersburg, MD).

Isolation of GCDCs from tonsils. Tonsils were cut into small pieces and digested with collagenase IV and deoxyribonuclease I as described¹². The cells, collected after two rounds of enzymatic digestion, were pooled and centrifuged

through a 50% Percoll gradient (Pharmacia) for 20 min at 400g. CD3⁺ T cells, CD14⁺ monocytes and CD19⁺ and CD20⁺ B cells were removed from the resulting low-density cells by magnetic beads (sheep anti-mouse Ig-coated Dynabeads) and stained with mouse anti-CD4-PE-Cy5 (Immunotech), anti-CD11c-PE (Becton-Dickinson), anti-CD3-FITC and CD34-FITC (Immunotech), anti-CD20-FITC and anti-CD16-FITC (Becton-Dickinson) and anti-CD1a-FITC (Ortho). CD4⁺CD11c⁺CD3⁺CD20[−]CD1a[−] cells were isolated by cell sorting (over 97% purity).

Phenotypic analysis of isolated GCDCs. FITC-labelled mAbs for phenotyping of sorted cells were from Immunotech (CD4, CD13, CD33, CD34, CD8, CD11b, CD21, CD18, CD54, CD58 and CD38), Becton-Dickinson (CD11c, CD14, CD2, TCR $\alpha\beta$, MHC Class II DR and DQ, CD16 and CD71), Coulter (TCR $\gamma\delta$), Sigma (CD45, CD44), Dako (CD45RO, CD23, CD11a), Kallestad (F(ab')₂ fragments of goat serum anti-human Ig), Ortho (anti- κ and anti- λ chains), Pharmingen (CD35), Medarex (CD32, CD64) and CD40 (mAb 89, generated in our laboratory). Cells, incubated with antibody for 15 min at 4 °C, were analysed after one wash with a FACScan flow cytometer. For the biotinylated reagents CD45RO and CTLA-4-Ig (from P. S. Linsley), cells were incubated with FITC-streptavidin (Immunotech) and washed before analysis.

Cell cultures. 5×10^3 CD4⁺CD11c⁺ GCDCs or IgD⁺CD38[−] GC B cells isolated from the same tonsils were cultured with 5×10^4 blood allogeneic CD4⁺ T cells in round-bottomed 96-well culture plates in RPMI 1640 containing 10% human AB⁺ serum. After 5 days, cells were pulsed with 1 μ Ci ³H-thymidine for 8 h before collecting and counting. Tests were carried out in triplicate; in the figures, standard deviations are indicated by bars.

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Decreased apoptosis in the brain and premature lethality in CPP32-deficient mice

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PROGRAMMED cell death (apoptosis) is a prominent feature of the development of the immune and nervous systems^{1,2}. The identification of the *Caenorhabditis elegans* cell death gene, *ced-3*, as a prototype of the interleukin-1 β converting enzyme (ICE) protease family has led to extensive evidence implicating these enzymes in apoptosis^{3,4}. Among the ten or more members of the ICE protease family, CPP32/yama/apopain⁵⁻⁷ exhibits the highest similarity to CED-3 in both sequence homology and substrate specificity⁸. To analyse its function *in vivo*, we generated CPP32-deficient mice by homologous recombination. These mice, born at a frequency lower than expected by mendelian genetics, were smaller than

their littermates and died at 1–3 weeks of age. Although their thymocytes retained normal susceptibility to various apoptotic stimuli, brain development in CPP32-deficient mice was profoundly affected, and discernible by embryonic day 12, resulting in a variety of hyperplasias and disorganized cell deployment. These supernumerary cells were postmitotic and terminally differentiated by the postnatal stage. Pyknotic clusters at sites of major morphogenetic change during normal brain development⁹ were not observed in the mutant embryos, indicating decreased apoptosis in the absence of CPP32. Thus CPP32 is shown to play a critical role during morphogenetic cell death^{9,10} in the mammalian brain.

We designed a targeting vector that replaces a *HindIII* genomic fragment encoding a conserved motif (QACRG) with the neomycin resistance gene (*neo*) (Fig. 1a). The correct homologous event in embryonic stem (ES) cells was confirmed by Southern blot analysis (Fig. 1b), and the absence of CPP32 expression in homozygous animals was confirmed by reverse transcription-polymerase chain reaction (RT-PCR) and western blot (Fig. 1c, d). Heterozygous mutant mice (CPP32^{+/-}) were healthy and normal in size. Interbreeding of heterozygous mice, however, produced a lower frequency (7%) of homozygous CPP32^{-/-} mice than expected from mendelian genetics, suggesting a degree of embryonic lethality of the null mutation phenotype. Furthermore, CPP32^{-/-} pups were considerably smaller than their littermates, and died between one and three weeks of age.

Thymi taken from two-week old CPP32^{-/-} mice yielded fewer thymocytes than those from wild-type littermates (120 million

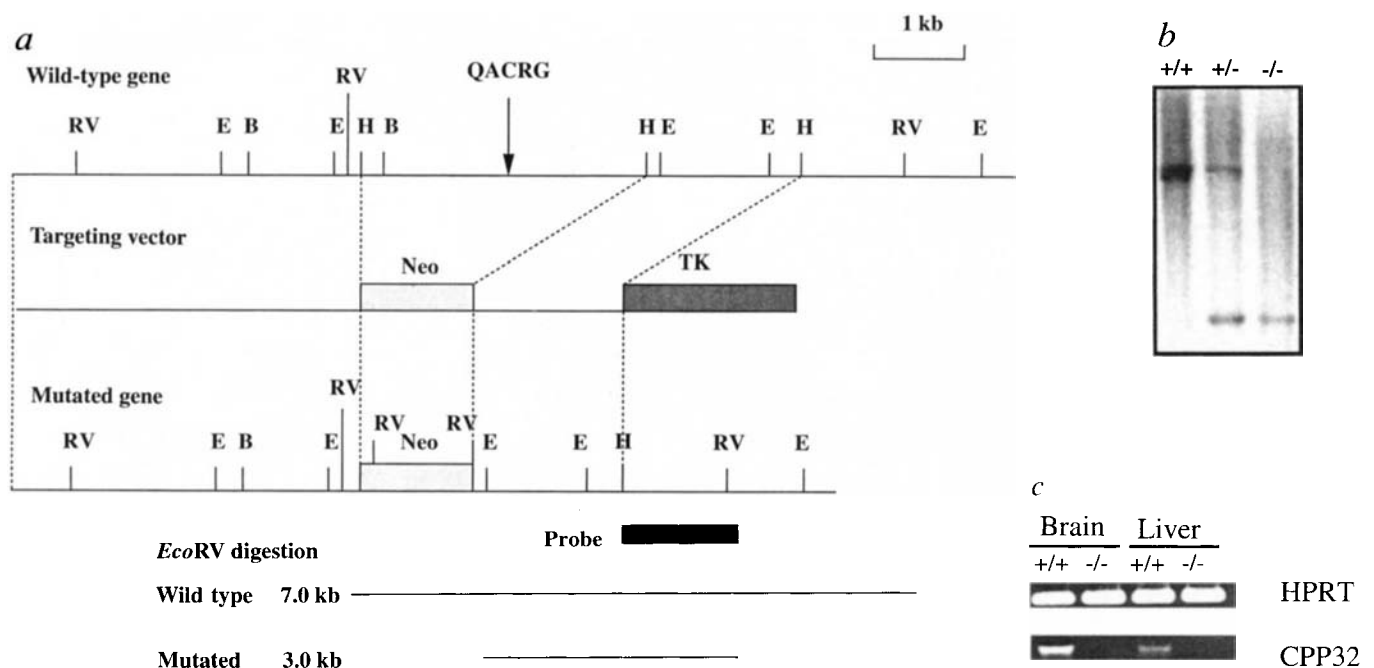


FIG. 1 Generation of CPP32 mutant mice by gene targeting. **a**, A schematic diagram of the *CPP32* locus, the targeting vector and the targeted allele. Restriction enzyme sites are indicated (B, *Bam*HI; E, *Eco*RI; H, *Hind*III; RV, *Eco*RV). QACRG is the pentapeptide motif of the catalytic site in CPP32 and is conserved among all the ICE/CED-3 family proteases. The arrow indicates a genomic region encoding this pentapeptide motif. **b**, Southern (DNA) blot showing correct targeting of the *CPP32* locus and genotyping of progeny. Genomic DNA samples were prepared from tails of the wild-type (+/+), heterozygous (+/-) and homozygous (-/-) mice, and were digested with *Eco*RV and probed as indicated. The resulting 7.0-kb and 3.0-kb bands correspond to the wild-type and mutated genotypes, respectively. **c**, RT-PCR showing the absence of *CPP32* message in -/- mutant mice. HPRT mRNA was used as internal control. **d**, Western blot showing the absence of *CPP32* protein in -/- mice. The 32K band corresponds to the *CPP32* precursor protein. The 30K band probably reflects a processed form of *CPP32* protein lacking its prodomain.

versus 200 million) in proportion to their smaller overall body size. Flow cytometric analysis of thymocytes with various cell-surface markers revealed normal CD4 and CD8 thymocyte populations (Fig. 2a) and normal expression patterns of developmental markers such as CD25, CD69 and heat-stable antigen (data not shown). Because thymocytes from ICE^{-/-} mice are resistant to anti-Fas antibody-induced apoptosis⁴, we examined the susceptibility of CPP32^{-/-} thymocytes to various apoptotic stimuli. To our surprise, thymocytes from CPP32^{-/-} mice and wild-type mice were equally sensitive to induction of apoptosis by anti-Fas antibody, dexamethasone, C2-ceramide, staurosporin (Fig. 2b) and γ -irradiation (data not shown). To test the possibility of functional compensation by other ICE-like proteases in the CPP32^{-/-} thymocytes, the cleavage of poly(ADP-ribose) polymerase (PARP) upon induction of apoptosis by staurosporin, dexamethasone or anti-Fas antibody was examined, there was a similar level of activity in CPP32^{-/-} and wild-type littermates (Fig. 2c). The apparently normal development and the susceptibility to apoptosis observed in CPP32^{-/-} thymocytes is therefore probably caused by other CPP32-like proteases, such as Mch2 and Mch3 (refs 11–13). Similarly, no discernible histological abnormalities were observed in the heart, lung, liver, kidney, spleen and testis, despite expression of CPP32 in these tissues in normal animals.

By contrast, the development of the nervous system was profoundly affected in all mice studied ($N = 16$), resulting in an array of abnormalities. Prominent protrusions of the brain tissue associated with skull defects were consistently noticed in the external

appearance of mutant mice (Fig. 3a). Unlike the smooth surface of the cerebrum mantle (agyria) in normal rodents (Fig. 3b), the CPP32^{-/-} mouse brain exhibited multiple indentations and ectopic cell masses (asterisk in Fig. 3c). Similarly, there were protrusions of the neuroepithelium in the retina, causing compression on the lens (filled arrow in Fig. 3e). Moreover, the protruding masses displayed the normal layering organization and all types of retinal neurons (Fig. 3f).

Ectopic cell masses were consistently located between the cerebral cortex, the hippocampus and the striatum (Fig. 3g). The laminar organization of the overlying cortex was obscured, possibly by supernumerary cells from the ectopic mass. The vast majority of cells within the ectopic mass were positively labelled by anti-microtubule-associated protein 2 (anti-MAP2) antibody, a neuronal marker (Fig. 3h). A small percentage of cells were also positively stained for the interneuron markers parvalbumin (Fig. 3i) and calbindin, or the astrocyte marker glial fibrillar associated protein (GFAP) (data not shown), which indicate the same neuronal and glial composition in the ectopic mass as the overlying cerebral cortex. Thus the ectopic mass with histological features of the cerebral cortex is remarkably similar to the recently reported 'double cortex' rat mutant (K. Lee, personal communication) and periventricular heterotopia in humans¹⁴. In contrast, bromodeoxyuridine (BrdU), injected intraperitoneally 24 h before the mice were killed, was incorporated into cells only in the subependymal zone (arrow in Fig. 3j), not in the ectopic masses, indicating that the supernumerary cells were not tumours

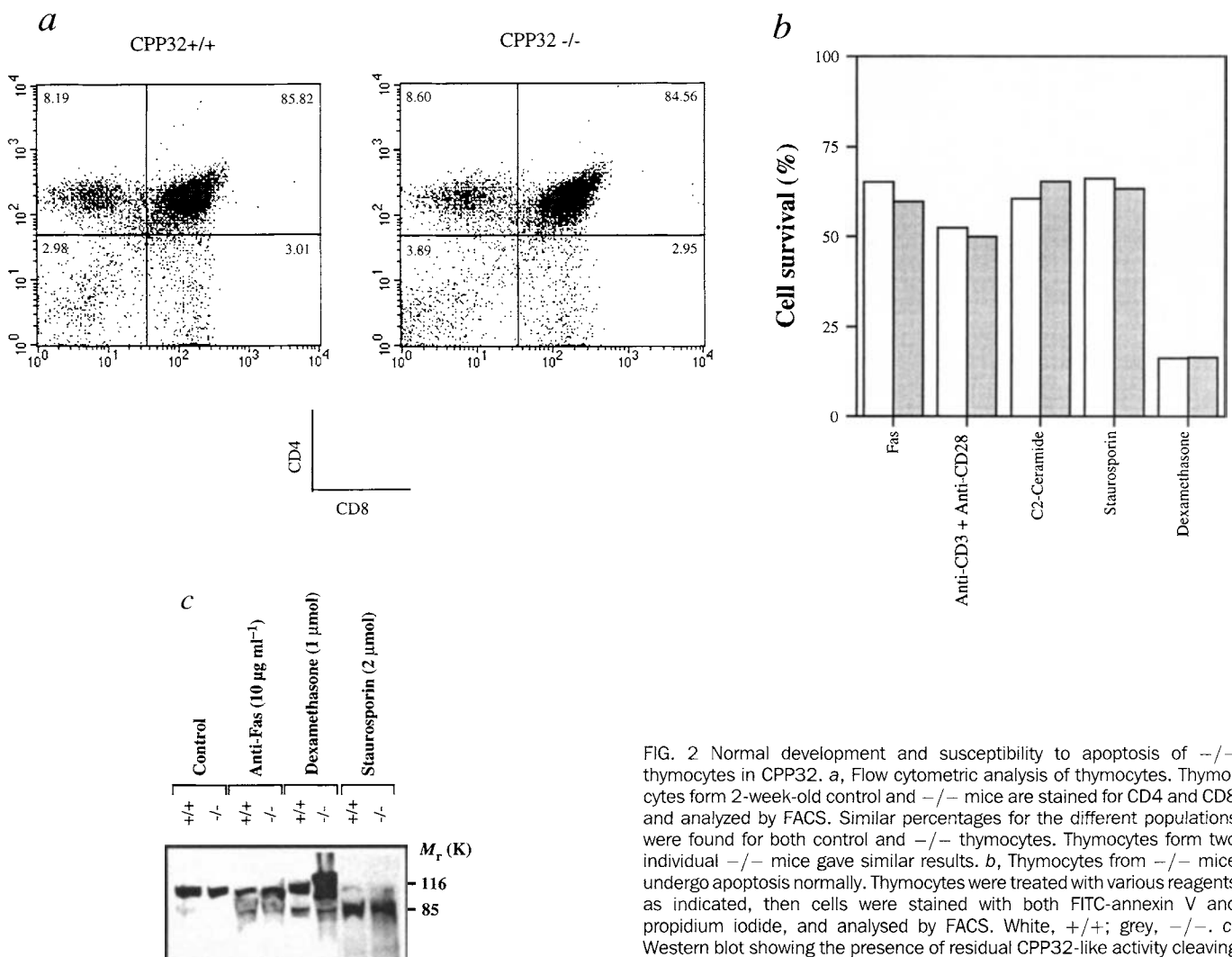


FIG. 2 Normal development and susceptibility to apoptosis of $-/-$ thymocytes in CPP32. **a**, Flow cytometric analysis of thymocytes. Thymocytes from 2-week-old control and $-/-$ mice are stained for CD4 and CD8 and analyzed by FACS. Similar percentages for the different populations were found for both control and $-/-$ thymocytes. Thymocytes from two individual $-/-$ mice gave similar results. **b**, Thymocytes from $-/-$ mice undergo apoptosis normally. Thymocytes were treated with various reagents as indicated, then cells were stained with both FITC-annexin V and propidium iodide, and analysed by FACS. White, $+/+$; grey, $-/-$. **c**, Western blot showing the presence of residual CPP32-like activity cleaving 110 K full-length PARP to 85 K.

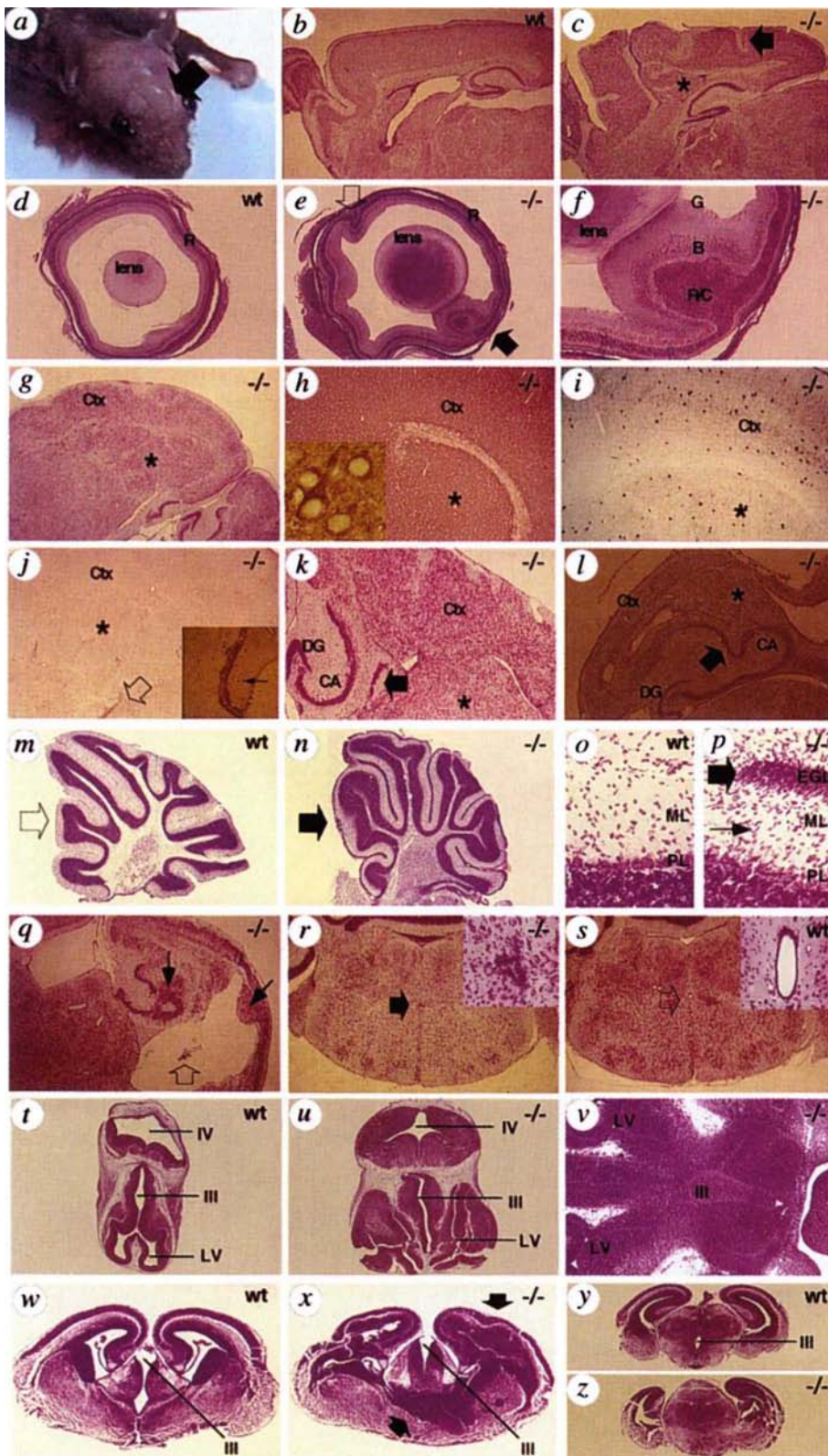


FIG. 3 The phenotype in $CPP32^{-/-}$ and wild-type (wt) animals. **a**, Spontaneous protrusion of brain tissue from the skull at the frontal bone area (arrow) in the $-/-$ mouse. **b**, **c**, Comparison of the cytoarchitectonic organization between wild-type (**b**) and $-/-$ (**c**) mice at postnatal day 16. Both sections were cut sagittally, stained with crystal violet, and photographed at the same magnification. The indentation of the cortex (arrow) and ectopic cell masses (asterisk) were present only in the mutant mouse. **d**, **e**, Cross-sections of the retina in the wild-type (**d**) and the mutant (**e**) mouse. Protrusion (filled arrow) and indentation (upper arrow) of the retinal neuroepithelium (R) were present in the mutant mouse. **f**, Higher-power photomicrograph of the protrusion in the mutant mouse retina showing normal layering including rods and cones (R/C), and bipolar (B) and ganglion cells (G). **g**–**j**, The cerebral cortex (Ctx) and adjacent ectopic cell masses (asterisk) have similar cellular composition including typical pyramidal neurons (Nissl's stained in **g**; stained by anti-MAP2 in **h**; insert shows a magnified view of labelled cells), and interneuron (stained by anti-parvalbumin in **i**). The absence of incorporation of BrdU (**j**) in the ectopic cell masses indicates they are not proliferative tumour cells. As an internal control, BrdU labelling in the subependymal zone (arrow) is shown in the insert figure. **k**, **l**, Ectopic cell masses (asterisk) were typically located between the cortex (Ctx) and the hippocampus (CA, cornu ammonis; DG, dentate gyrus) forming an additional secondary Ammon's horn or an extended convex-shaped pyramidal cell layer (arrows). **m**–**p**, Comparison of the cerebellar cortex between wild-type (**m**, **o**) and mutant (**n**, **p**) mouse littermates at postnatal day 16. The existence of the external granule layer (EGL, arrow) was found only in the mutant mouse. Note numerous spindle-shaped granule cells (small arrow) across the molecular layer (ML) on their way to the internal granule layer situated beneath the Purkinje cell layer (PL). **q**–**s**, Normal-sized choroid plexus (thick arrows) and ectopic cell masses (small arrows) in the hydrocephalic brain. The hydrocephaly was caused by an obstruction of the cerebral aqueduct by supernumerary cells (arrow and insert) in the mutant mouse. **t**–**v**, Wild-type (**t**) and mutant (**u**, **v**) embryos at embryonic day 12, photographed at the same magnification to show thicker cerebral wall. Lateral (LV) and third (III) and fourth (IV) ventricles were labelled for orientation. These ventricular lumen were considerably narrowed in the mutant embryo. **w**–**z**, Comparison of the mutant and normal embryos at embryonic day 17. Thicker and disorganized germinal zones (arrow) were seen in the mutant embryo (**x**, **z**) with the initial formation of the cortical indentations (arrow). The obstruction of the third ventricle was also prominent in this mutant embryo. Magnification: **b**, **c**, **g**, **q**, **r**, **s** $\times 25$; **d**, **e**, **j**, **k**, **l**, **m**, **n**, **t**, **u**, **v**, **w**, **x**, **y**, **z** $\times 40$; **f**, **h**, **i** $\times 100$; **o**, **p** $\times 400$.

or undifferentiated proliferative epithelial cells.

The supernumerary cells were even capable of assuming proper cellular deployment. For example, a second partial Ammon's horn (arrow in Fig. 3k) and the expanded, convex-shaped pyramidal cell layer of the hippocampus were observed (arrow in Fig. 3l) juxtaposed to the ectopic masses (asterisk in Fig. 3k). The incorporation of supernumerary cells into normal nervous tissue was also found in the cerebellum. Cerebellar granule cells are

generated along the surface of the cerebellar hemisphere, and migrate across the molecular layer into the internal granule layer situated beneath the Purkinje cell layer^{15,16}. The germinal layer in the wild-type mouse had been virtually exhausted by postnatal day 16 when it was killed (Fig. 3m), but in the knockout littermate it remained thick and mitotically active (Fig. 3n). In addition, numerous spindle-shaped granule cells were observed traversing the molecular layer in the mutant mouse (Fig. 3p). Finally, the

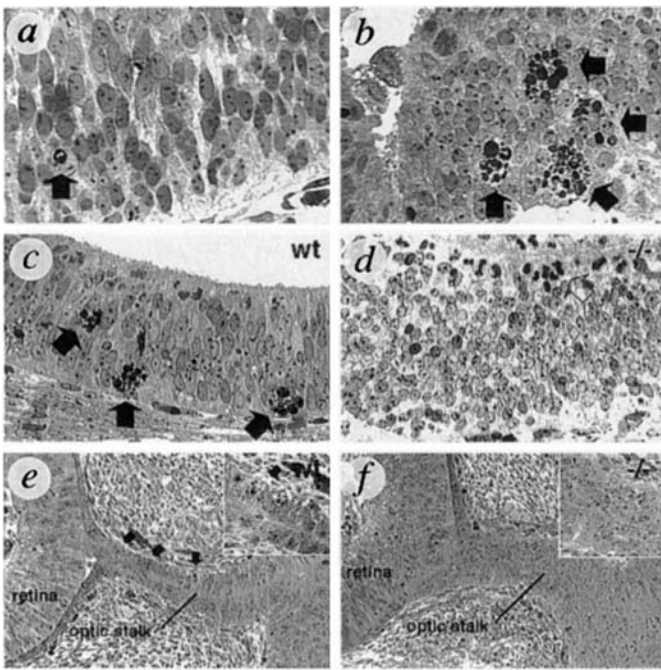


FIG. 4 Distribution of pyknotic cells in the $CPP32^{-/-}$ and wild-type control embryos. Dying cells in the normal embryonic brain appear either as isolated scattered pyknotic figures (a, arrow) or as pyknotic clusters (b) that are located in the areas undergoing major morphogenetic changes⁹. c, d, Comparison of sections across the median–dorsal wall of the diencephalon reveals prominent pyknotic clusters in the wild-type (c) but not in the mutant (d). There is also increased cell density with many mitotic figures (unfilled arrow) along the ventricular wall in the $-/-$ embryo. e, f, Comparison of the optic stalk in the wild-type (e) and mutant (f) embryos at embryonic day 12. The large number of pyknotic cells (arrows; insert shows magnified photomicrograph), characteristic of normal embryonic degeneration of the optic stalk¹⁹, were found only in the wild type (e). In contrast, the optic stalks in the $-/-$ embryo display fewer pyknotic cells and remain larger (f). Magnification: a–d and inserts in e and f, $\times 1,000$; e, $\times 400$.

thickness of the internal granular layer appears to be increased in the mutant mouse, indicating that the persistence of the germinal layer was due to continued production of granule cells rather than migratory defects.

In some cases the existence of supernumerary cells caused secondary effects on other brain structures. For example, in one $CPP32^{-/-}$ mouse hydrocephalus with elevated intracranial pressure may have played and thinned the cerebral cortex (Fig. 3g). The hydrocephalus in this mouse was probably caused by the obstruction of the cerebral aqueduct by the excessive cellularity of the brainstem (Fig. 3r, s). Therefore slight variation in the temporal or spatial appearance of the supernumerary cells can cause profoundly different developmental consequences, contributing to the individually variable expressivity of the observed $CPP32^{-/-}$ phenotype.

To investigate the mechanism underlying the $CPP32^{-/-}$ phenotype, we examined both mutant and wild-type embryos during development. At embryonic day 12, the gross brain abnormality was prominent enough to be identified by visual inspection of the embryos even before genotyping. The percentage of homozygous mutant embryos obtained from interbreeding of heterozygous mice was compatible with mendelian genetics (4 of 14). At this stage, the structural abnormality was observed mainly as increased in cell number and density of the brainstem (Fig. 3t–v). That these abnormalities were discernible at such an early developmental stage suggests that the discrepancy of the ratio of homozygous mutants between litters of newborn pups and embryos is due to the death of the most severely affected $CPP32^{-/-}$ embryos.

Because pyknosis is a prominent feature of apoptosis¹⁷, we examined serial 1- μ m semi-thin sections of $CPP32^{-/-}$ and wild-type embryonic brains at embryonic day 12 for the presence of pyknotic nuclei. Both $CPP32^{-/-}$ and wild-type embryos had a few scattered pyknotic cells at this stage (Fig. 4a). The low incidence of such sporadic pyknotic cells, as well as the increased cellularity of the mutant embryo, had hampered a definitive quantitative comparison. However, although the clusters of pyknotic cells were clearly present at the interventricular junction (Fig. 4b) and the caudal–medial wall of the diencephalon in the wild-type embryo (Fig. 4c), they were absent in the mutant. Instead, a thicker diencephalic wall with supernumerary cell masses was observed in the $CPP32^{-/-}$ embryo (Fig. 4d).

The existence of clusters of pyknotic cells had been reported in the spinal cord¹⁸, optic stalk¹⁹ and the fusion of the cerebral hemispheres²⁰ during normal neurogenesis. These pyknotic clusters have been attributed to morphogenetic cell death as a result of their temporally and spatially specific presence and the simultaneously massive apoptosis in a restricted region during embryogenesis⁹. Another site of active pyknotic clusters during normal development at this stage is the optic stalk¹⁹, at which they were clearly present in the wild-type embryos (Fig. 4e). In contrast, the mutant optic stalk had significantly fewer pyknotic cells and was much larger than the normal. Therefore the absence of pyknotic clusters in the mutant embryo indicates that the phenotype of $CPP32$ knockout is caused primarily by a decrease of apoptosis. Five days later, at embryonic day 17, the decreased apoptosis of progenitor cells is reflected in thicker and disorganized germinal zones forming the basis of the ‘double cortex’ seen at the postnatal stage (Fig. 3w, x). Moreover, in the mutant embryo the diencephalic lumen was frequently obstructed, resulting in developmental complications such as hydrocephalus (Fig. 3y, z).

These studies have several implications. First, the apoptotic defect, as well as the postmitotic and well-differentiated properties of supernumerary cells seen in the $CPP32^{-/-}$ mouse, shows some similarities to the consequences of the *ced-3* mutation in *C. elegans*²¹. Moreover, both mutations primarily affect neuronal cells²². To our knowledge, we have demonstrated for the first time that mutation of a mammalian homologue of *ced-3* leads to decreased cell death and a supernumerary cell population during development, suggesting that the basic machinery of programmed cell death is conserved evolutionarily. Second, the restricted phenotype raises the possibility that other members of the ICE protease family may also have an important role during apoptosis in other tissues, cell types or in response to cell-death triggers. Ten mammalian homologues of *ced-3* have been identified so far, and they are coexpressed in many tissues and cell types³, suggesting possible redundancy of the cell-death pathways in complex biological systems. However, the $CPP32^{-/-}$ phenotype is intriguingly brain-specific, despite the fact that Mch2 and Mch3, the two most closely related CED-3 subfamily protease²³, were readily detected by western blot in all major subdivisions of the nervous system (data not shown). Finally, our results indicate a new aspect of neuronal cell death during early development. According to the generally accepted scheme, there are three major types of cell death during development: phylogenetic, morphogenetic and histogenetic¹¹, of which the epigenetic influence on neuronal apoptosis elucidated in previous studies^{24–26} represents the process of histogenetic cell death². Our observations provide evidence that $CPP32$ plays an important role in another aspect of neuronal apoptosis, namely the early morphogenetic cell death in the central nervous system. Its possible effect on the other types of apoptosis will be the subject of future studies. □

Methods

Generation of $CPP32$ knockout mice. The $CPP32$ gene fragments were cloned from 129/Sv liver DNA genomic library (Stratagene). A 4.5-kb *NotI*–*HindIII* fragment was cloned into Bluescript II SK (Stratagene) together with a neomycin-resistance cassette (PGK-neo). The resulting plasmid was

digested with *NotI* and *Clal* and subcloned into a thymidine kinase gene vector with a 1.6-kb *Clal*-*Bam*HI fragment from a plasmid containing an *Eco*RI fragment encoding the 3' end of the CPP32 gene to generate the targeting construct. The targeting construct was introduced into W9.5 embryonic stem cells. Transfectants resistant to G418 (Gibco) and gancyclovir were isolated and screened by Southern blot analysis. The efficiency of homologous recombination was 1 in 26 clones. After injection into blastocysts, one of the targeted clones transmitted the targeted allele. Heterozygous mice (129 × B6F1 genotype) were interbred to generate homozygous mice.

RT-PCR analysis. Total RNAs were prepared from brain and liver using TRIzol Reagent (Life Technologies). The first strand of cDNA was obtained by standard reverse transcription. The 5' primer (5'-CTCTTCATCATTCAGGCTGCCGTGGTAC-3') and 3' primer (5'-CCTCTAGTGATAAAGTACAAGTTC-3') used in PCR correspond to the conserved QACRG region and the C terminus, respectively. A 368-bp band corresponding to the CPP32 message was amplified.

Western blot analysis. Whole-cell lysates prepared from CHO cells, CPP32^{+/+} and CPP32^{-/-} thymocytes were analysed by 14% SDS-PAGE (Novex) and immunoblotted with a rabbit polyclonal antibody (1:2,000) raised against amino acids 12–20 at the N terminus of the hamster CPP32 (SCA)²⁷. For a study of PARP cleavage, whole-cell lysates from thymocytes treated with various reagents as described were analysed by 12% SDS-PAGE and immunoblotted with anti-PARP (1:2,000) antiserum. All immunoblots were visualized by ECL (Amersham).

Viability assay. Thymocytes were prepared from two-week-old control and CPP32^{-/-} mice. Cells (1×10^6) were resuspended in RPMI (10% FBS) medium and subjected to the following treatments at 37 °C: anti-Fas antibody (Jo2 and RK8, $1 \mu\text{g m}^{-1}$) for 24 h, immobilized antiCD3 (2C11, $20 \mu\text{g m}^{-1}$) plus anti CD28 ($20 \mu\text{g ml}^{-1}$, PharMingen) for 24 h, C2-Ceramide ($70 \mu\text{mol}$, Sigma) for 4 h, Staurosporin ($2 \mu\text{mol}$, Sigma) for 4 h and Dexamethasone ($1 \mu\text{mol}$, Sigma) for 4 h. Half of the cells were then washed and stained with both FITC-annexin V (BioWhittaker) and propidium iodide, and analysed by fluorescence-activated cell sorting (Becton Dickinson). The percentage of surviving cells was calculated from the percentage of FITC-negative populations on the histograms^{28,29}. From each sample, 1×10^5 events were collected.

Histology and immunocytochemistry. Postnatal animals were fixed by transcardial perfusion; embryos were fixed by immersion in 4% paraformaldehyde. The brains were dissected and embedded in paraffin, cut serially at 10 μm and stained by 0.1% crystal violet or processed for BrdU³⁰, GFAP, MAP2 or parvalbumin immunohistochemistry. For semi-thin sections, embryos were fixed in a mixture of 4% paraformaldehyde and 1.5% glutaraldehyde, embedded in plastic and serial, 1- μm sections stained by 1% toluidine blue. All comparisons between wild type mutants were performed on age-matched littermates.

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A death-domain-containing receptor that mediates apoptosis

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THE cell-killing effects of the cytokines TNF- α and FasL are mediated by the distinct cell-surface receptors TNFR1, TNFR2 and Fas (also known as CD95/APO-1), which are all members of a receptor superfamily that is important for regulating cell survival^{1–4}. The cytoplasmic regions of TNFR1 and Fas contain a conserved 'death' domain which is an essential component of the signal pathway that triggers apoptosis and activation of the transcription factor NF- κ B (refs 5, 6). Here we report the isolation of a 54K receptor that is a new member of the TNFR superfamily, using the death domain of TNFR1 in a yeast two-hybrid system^{7,8}. This protein, WSL-1, is most similar to TNFR1 itself, particularly in the death-domain region. The gene *wsl-1* is capable of inducing apoptosis when transfected into 3T3 and 293 cells, and can also activate NF- κ B in 293 cells. Like TNFR1, WSL-1 will homodimerize in yeast. WSL-1 also interacts specifically with the TNFR1-associated molecule TRADD⁹. The tissue distribution is very restricted and significantly different from that of Fas and TNFR1.

In an attempt to isolate proteins that interact with TNFR1, we cloned the death domain of this receptor and expressed it in yeast as a GAL4 fusion protein. This construct was used to screen a HeLa cell two-hybrid library^{7,8}. From approximately two million transformants, seven clones were isolated that interacted specifically with the TNFR1 death domain. Six of these clones encoded portions of the cytoplasmic region of TNFR1 (refs 10, 11). The seventh clone, however, was found to encode a new complementary DNA which we have called *wsl-1*. This cDNA, identified in the two-hybrid experiment, did not contain a complete open reading frame (ORF). A cDNA encoding the complete ORF for *wsl-1* was cloned by 5' RACE PCR (for rapid amplification of cloned ends by polymerase chain reaction). Several clones were isolated and were shown to encode a protein of 417 amino acids (Fig. 1a).

Database searches identified the protein as a new member of the TNFR superfamily, containing four extracellular regions with the characteristic cysteine-rich motif¹. WSL-1 is most similar in sequence to TNFR1 itself (28% identity): this similarity is particularly striking between the carboxyl terminus of WSL-1 and the TNFR1 death domain (45% identity; Fig. 1b). The death domain of WSL-1 is more closely related to TNFR1 than the TNFR1-associated death-domain-containing protein TRADD (32%)⁹.

Besides the cDNA encoding the full-length receptor, two predicted alternative splice variants were identified by polymerase chain reaction with reverse transcription (RT-PCR) of poly(A)⁺ thymus RNA. The alternative splicing occurs 5' to the transmembrane domain. These variants (Fig. 1a) encode soluble forms of the *wsl-1* extracellular domain of either 218 or 253 amino acids. Northern analysis using *wsl-1* cDNA as a probe revealed a single 3.4-kilobase (kb) transcript (Fig. 2), expressed in spleen, thymus and peripheral blood lymphocytes. No expression was detected in heart, brain, placenta, lung, liver, skeletal muscle, kidney or pancreas. A 54K protein was detected by western blotting of total spleen protein by using polyclonal sera raised against an intracellular peptide sequence (data not shown).

FIG. 1 Sequence of human *ws1-1*. *a*, Predicted amino-acid sequence of human *ws1-1*. The putative transmembrane domain is boxed and the death domain is underlined. Splice junctions are indicated by filled triangles. The nucleotide sequence data will appear in the EMBL, GenBank and DDBJ nucleotide sequence databases under the accession number Y09392. *b*, Homology in the death domain region between human WSL-1, human TRADD and human TNFR1. Amino acids 335–413 of WSL-1 are compared with amino acids 222–287 of TRADD and amino acids 359–438 of TNFR1. Asterisks above the sequence indicate residues where mutation to alanine has been shown to inactivate TNFR1 signalling⁵.

a

```

1  MEQRPRGCAA VAAALLLVLL GARAQGGTRS PRCDGAGDFH KKIGLFCCRG
51  CPAGHYLKAP CTEPCGNSTC LVCPQDTFLA WENHHNSECA RCQACDEQAS
101 QVALENCNAV ADTRCGCKPG WFVECVQVSC VSSSPFYCQP CLDCGALHRH
151 TRLLCSRRTD DCGTCLPGFY EHGDGCVSCP TSTLGSCPER CAAVCGWRO*
201 FWVQVLLAGL VVPLLLGATL TTYTRHCWPH KPLVTAEAG MEALTPPPAT
251 HLSPLDSAHT LLAPPSSEK ICTVQLVGNV WTPGYPETQE ALCPQVTWSW
301 DQLPSRALGP ARAPTLSPEP PAGSPAMMLQ PGPQLYDVM AVPARRWKEF
351 VRTLGLREAE IEAVEVEIGL FRDQYEMLK HWRQQPAGL GAVYAALERM
401 GLDGCVEDLR SRLQRGP

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b

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TRADD      L Y A V V E N V P P L R W K E F V R R L G L S D H E I D R L E L Q N G C R I A L R D P A L D S L A Y E Y E
TNFR1      L Y D V M D A V P A R R W K E F V R T L G L R E A E I E A V E I V E I G L R I E A Q Y S M L A T W R R
WSL-1      L Y D V M D A V P A R R W K E F V R T L G L R E A E I E A V E I V E I G L R I E A Q Y E M L K H W R Q

TRADD      R E G L Y E Q A F Q L L R R F V Q A E G R R A T L Q R L V V - -
TNFR1      R T P R R R E A T L E L L G R V L R D M D L L G C L E D I E - - -
WSL-1      Q Q P - - A G L G A V Y A A L E R M G L D G C V E D L R S R L Q

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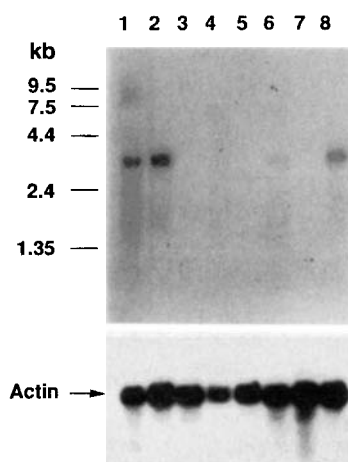


FIG. 2 Northern blot analysis of *ws1-1* expression (human II). Lanes: 1, spleen; 2, thymus; 3, prostate; 4, testis; 5, ovary; 6, small intestine; 7, colon; 8, peripheral blood leukocytes.

Overexpression of TNFR1 or of its death domain alone will trigger a cytotoxic effect and activate NF- κ B (refs 5, 10, 12). We therefore investigated whether WSL-1 is also capable of inducing these responses. To determine whether apoptosis is induced by *ws1-1*, 3T3 cells and 293 cells were transfected with full-length *ws1-1*, a construct expressing a truncated form of *ws1-1* lacking the cytoplasmic region, or a construct expressing Myc-tagged intracellular domain. A significant amount of cell death of cells transfected with *ws1-1* followed within 24–48 hours, but not in cells transfected with the truncated form. The transfected cells showed the characteristic morphology associated with apoptosis (Fig. 3*B*). This is consistent with the observation that overexpression of full-length TNFR1 can induce cell death¹⁰. Furthermore, transfection of cells with a vector expressing the cytoplasmic portion of WSL-1 with an N-terminal Myc epitope tag demonstrated that cells expressing Myc-tagged WSL-1 were undergoing apoptosis (data not shown).

To determine the effects of *ws1-1* on NF- κ B activation, the cDNA was cloned into a mammalian expression vector and co-

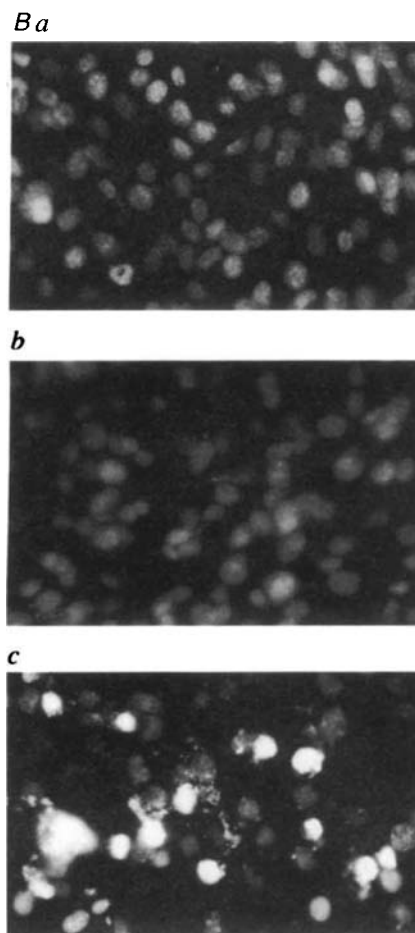
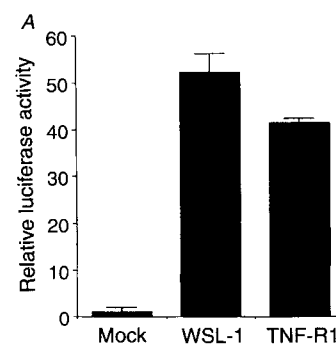


FIG. 3 *A*, Effect of *ws1-1* on NF- κ B activation. The level of activation in a luciferase reporter gene assay induced by overexpression of *ws1-1* is compared with that induced by either *tnfr1* or empty vector. *B*, *ws1-1* induction of apoptosis: *a*, untransfected cells; *b*, cells transfected with a 253-amino-acid truncated form of *ws1-1* lacking the intracellular domain; *c*, cells transfected with a construct expressing *ws1-1*.

transfected with a reporter construct containing an NF- κ B response element coupled to the luciferase gene. As shown in Fig. 3a, expression of WSL-1 is sufficient to induce activation of NF- κ B, comparable to that produced by overexpression of TNFR1 itself.

The death domain seems to modulate multimeric interactions between receptor-associated proteins that can induce apoptosis^{9–11,13–15}. We next investigated whether WSL-1 could homodimerize, or interact with molecules known to be components of the TNFR1 signalling pathway. Using the yeast two-hybrid system, no binding was detected to RIP, FADD, TRAFs 1–3 (ref. 1) or TRAF-6 (ref. 16); neither did WSL-1 interact with the Fas antigen. However, in addition to its heterodimeric interaction with the TNFR1 death domain, WSL-1 can interact strongly with itself (Fig. 4a), and with the TNFR1-associated molecule TRADD⁹, a feature also characteristic of the TNFR1 death domain^{10,11}. This specific interaction with TRADD was confirmed by co-immunoprecipitation (Fig. 4b). Several point mutations have been identified in the TNFR1 death domain that block its

ability to dimerize and trigger NF- κ B activation and cytotoxicity.^{5,11} Again using the two-hybrid system, we investigated whether similar mutations could affect the ability of WSL-1 to interact with TNFR1 or to homodimerize. As shown in Fig. 4a, WSL-1 carrying any one of three point mutations (Fig. 1b) did not homodimerize or interact with TNFR1. Furthermore, in contrast to wild-type WSL-1, the full-length receptor containing point mutation L354A (alanine substituted for leucine at residue 354) failed to induce apoptosis. The significant conservation of these residues between TNFR1, TRADD and WSL-1 suggests that they are critical for mediating protein–protein interactions (Fig. 1b). Our results indicate that WSL-1 uses signalling pathways similar to TNFR1, with TRADD being a common interacting molecule that regulates NF- κ B activation and/or apoptosis. It is not clear whether TNF- α itself, Fas ligand (FasL) or TRAIL¹⁷ are ligands for WSL-1, or indeed whether the ligand is different, as we have not yet been able to establish a cell line that stably expresses *wsl-1*.

The immediate post-receptor signalling mechanisms used by TNFR-1 and Fas have become clearer since the discovery of several cytoplasmic proteins that can interact with and transmit signals from these receptors^{2,9,10,13–15}. These proteins share sequence similarity, particularly in the death domain, and most of them induce apoptosis when overexpressed. We have cloned a new member of the TNFR superfamily which, like Fas and TNFR-1, contains a death domain and can trigger rapid apoptosis. This protein can also activate NF- κ B and binds specifically to the TNFR1 signal-transducing molecule TRADD.

TNFR-1 is expressed in most cell types, consistent with the many functions of TNF. In contrast, expression of human Fas is tissue-specific¹⁸. Expression of *Wsl-1* is also restricted, but the distribution of WSL-1 is different from that of Fas, being predominant in spleen, thymus and peripheral blood lymphocytes. This indicates that *wsl-1* probably has a different role from that of TNFR-1 and Fas in the regulation of apoptosis. □

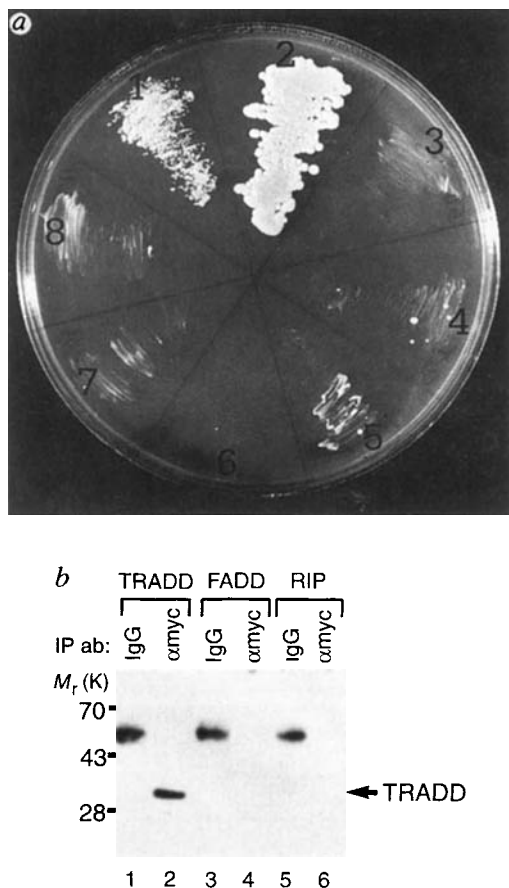


FIG. 4 a, Interactions between WSL-1, WSL-1 point mutants and TNFR1 death domain (dd). 1, WSL-1 compared with TNFR1 dd; 2, WSL-1 and WSL-1; 3, mutL365A and WSL-1; 4, mutL356A and TNFR1 dd; 5, mutL354A and WSL-1; 6, mutL354A and TNFR1 dd; 7, mutD373A and WSL-1; 8, mutD373A and TNFR1. Positive interactions are seen only with WSL-1 homodimers and WSL-1/TNFR1 heterodimers. Point mutations L354A, L356A and D373A correspond to substitutions of amino-acid residues 354, 356 and 373, respectively, of human WSL-1 with alanine. b, Immunoprecipitation (IP) showing a specific *in vivo* association of WSL-1 with TRADD. 293 cells were transfected with the indicated combinations of expression vectors for Myc-tagged *wsl-1* and Flag-tagged FADD²¹, TRADD⁹ or RIP²². After 24 h, extracts were immunoprecipitated with an anti-Myc monoclonal antibody (α) or control mouse IgG as described⁹. Immune complexes were separated by SDS-PAGE and analysed by immunoblotting with anti-Flag monoclonal antibody.

Methods

cDNA cloning. Coding sequence comprising nucleotide residues 1,268–1,532 of human TNFR1 was isolated by PCR and cloned in-frame with the GAL4-binding domain into pASCYH2, a derivative of pAS1 (ref. 19). This was co-transformed with 2×10^6 cDNAs from a HeLa cell library (Clontech) into yeast Y190, a derivative of yeast Y153 (ref. 7). The transformants were plated onto SD medium lacking histidine, tryptophan and leucine in the presence of 25 mM 3-aminotriazole (Sigma). After incubation at 30 °C for 10 days, the colonies were screened for LacZ activity using a freeze–fracture assay⁷. Putative interacting clones were back-extracted from yeast¹⁹ and rescreened. Secondary positives were then sequenced using sequenase (US Biochemicals); the resulting sequences were used to scan the GenBank and EMBL databases for homologous sequences using the FASTA program. cDNAs containing the complete ORF were isolated using 5' RACE PCR²⁰ (Gibco BRL) performed on human thymus mRNA (Clontech). The WSL-1 protein sequence was aligned against human TRADD and human TNFR1 death domains using the Geneworks sequence-analysis package. The multiple-tissue northern blot (Clontech) was hybridized with a ³²P-labelled intracellular fragment of *wsl-1*. The blot was exposed to Kodak XAR-5 film with an intensifying screen for 9 days at –70 °C. Hybridization with actin is shown as an internal control for loading (3 days exposure with an intensifying screen at –70 °C).

Transient transfections. 293 cells were transfected with expression vectors for either *wsl-1* or *tnfr1* (ref. 5) together with the pLAM-luc reporter as described⁹. After 36 h, luciferase activities were determined. Values represent luciferase activities relative to cells transfected with the empty expression vector pRK5 and are shown as mean $\pm$ s.e.m. for experiments done in duplicate. Mouse fibroblast 3T3 cells (1×10^5) were transfected with 2 μ g of an expression vector (pCDNA3, Invitrogen) containing full-length *wsl-1* or a truncated *wsl-1* using 10 μ l of the transfection reagent DOTAP (Boehringer Mannheim) in DMEM medium containing 5% FCS. Cells were fixed 26 h after transfection with 4% formaldehyde and stained with propidium iodide (1 μ g ml^{–1}, 100 μ g ml^{–1} RNase) for 20 min at 37 °C.

Mutagenesis. Mutations were introduced using a Sculptor *in vitro* mutagenesis system (Amersham). Yeast Y190 cells were transformed with pACT and pASCYH2 vectors⁷ expressing wild-type or mutants L356A, D373A or L354A WSL-1, and the TNFR1 death domain. Cells were grown for 2 d at 30 °C on SD medium lacking tryptophan, leucine and histidine in the presence of 25 mM 3-aminotriazole (Sigma). Growth indicates protein–protein interaction.

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Enhanced processivity of RNA polymerase II triggered by Tat-induced phosphorylation of its carboxy-terminal domain

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THE protein Tat is encoded by the HIV-1 genome and is essential for viral replication because of its activation of viral transcription. Tat enhances the ability of RNA polymerase II (Pol II) to move long distances down the DNA through a poorly understood mechanism that involves its binding to the 5' end of the nascent HIV-1 transcript^{1–5}. It has been suggested^{6–10} that the stimulation of transcript elongation by conventional DNA-binding activators may involve phosphorylation of the carboxy-terminal domain (CTD) of Pol II by the transcription factor TFIIF^{11–13} through the associated CAK kinase^{14–16}. Here we show that Tat-enhanced HIV-1 transcription *in vitro* requires both TFIIF and the CTD of Pol II. In addition, Tat, through its activation domain, both interacts with a functional TFIIF-containing complex and stimulates phosphorylation of a CTD-containing substrate by the TFIIF kinase. Under conditions that jointly restrict transcriptional elongation^{5,17} and TFIIF-mediated CTD phosphorylation, Tat stimulates both these activities. Furthermore, RNA synthesis is required for Tat to stimulate phosphorylation of the CTD when it is part of an initiation complex, as expected from Tat's interaction with viral transcripts¹. Thus, stimulation of Pol II elongation by Tat may involve direct effects on TFIIF-mediated CTD phosphorylation.

To examine a potential role of the TFIIF CTD kinase activity in Tat effects on processivity, we used nuclear extracts that had been treated with citrate to lower the intrinsically high HIV-1 transcription activity. In citrate-treated extracts, but not standard extracts, Tat enhances transcription from the HIV-1 template in a manner dependent upon an intact Tat-binding site (TAR) (Fig. 1a; refs 5, 17). Consistent with the demonstration that citrate treatment selectively reduces elongation (and not initiation) on HIV-1 templates^{5,17}, other inhibitors of elongation (the kinase inhibitors H-8 and DRB)^{4,7} also reduced HIV-1 transcription activity (Fig. 1a, and data not shown). Although the H-8 and DRB effects were not reversible by Tat, the common effect of citrate, H-8 and DRB on elongation indicates that citrate, like H-8 and DRB (ref. 7), may inhibit phosphorylation of the Pol II CTD by TFIIF.

To test the suggested effect of TFIIF on Tat function, we complemented TFIIF-depleted, citrate-treated extracts with Tat alone or with Tat plus TFIIF preparations of variable purity. All

four TFIIF fractions tested, including highly purified preparations uncontaminated by other general factors, showed synergistic effects with Tat (Fig. 1b) (the residual transcription activity with Tat alone may reflect residual TFIIF activity in the extract). Although the level of Tat-activated HIV-1 transcription that is TAR RNA dependent was similar for all TFIIF fractions tested, there was a greater stimulation of HIV-1 transcription from both wild-type and mutant TAR RNA templates when a TFIIF–TFIIF–TFIIF fraction was used. This suggests that factors in addition to TFIIF have been depleted from the extract, or that

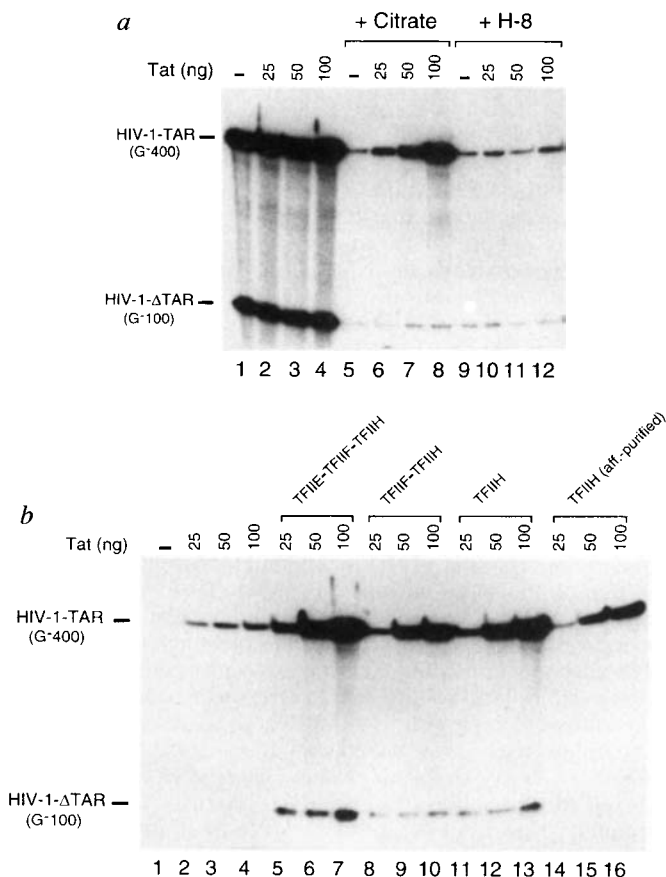


FIG. 1 Tat-enhanced HIV-1 transcription *in vitro*. a, TAR-dependent Tat function in citrate-treated nuclear extracts. b, Tat function in TFIIF-depleted citrate-treated extract in response to addition of TFIIF fractions. TFIIF fractions were added at saturating amounts that support maximal HIV-1 activated transcription by Sp1 (data not shown). The reaction products (in a and b) reflect specifically initiated transcripts derived from wild-type or mutated TAR element HIV-1 templates with 400-nt and 100-nt G-less cassettes, respectively²⁸.

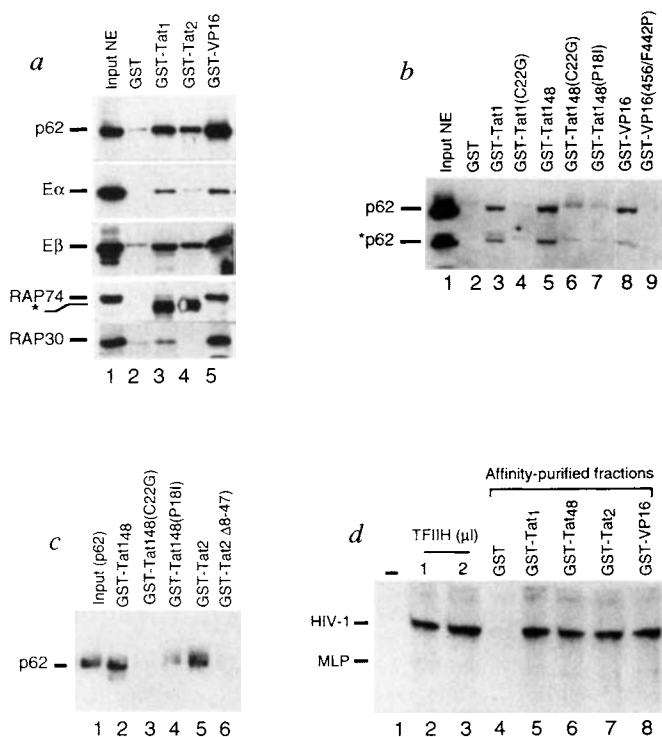


FIG. 2 Interactions between Tat and general transcription factors. *a*, Interaction of Tat with a TFIIE–TFIIF–TFIIH complex(es) in nuclear extract. *b*, Interaction of activation-competent versus activation-defective Tat proteins with TFIIF in nuclear extracts. E α /E β , RAP30/RAP74 and p62 are subunits of TFIIE, TFIIF and TFIIH, respectively. Inputs in *a* and *b* contained 5 μ l of nuclear extract. *RAP74 and *p62 represent degradation products of the corresponding factors. *c*, Specific interaction of recombinant TFIIH(p62) with activation-competent Tat proteins. Input, 10 ng p62. *d*, Interaction of transcriptionally active TFIIH with Tat and VP16. Transcription reactions¹⁷ containing 5 μ l TFIIF-depleted HeLa nuclear extracts, 100 ng HIV-1-TAR-G400 and 50 ng AdML promoter (MLP-G-200) were complemented with the indicated amounts of standard affinity-purified TFIIH fraction (lanes 2 and 3), or with 1 μ l each of the affinity-purified fractions bound (in nuclear extracts) to the indicated GST-fusion proteins.

this fraction may contain other activities that in part obviate the Tat requirement⁵.

Although initiation functions have been documented for TFIIE, TFIIF and TFIIH (refs 18, 19), TFIIF is also an elongation factor²⁰, with a possible role in Tat function⁵, and it has been suggested^{17,21} that TFIIE and TFIIH may also function at promoter clearance and/or elongation steps. Hence, we used immobilized GST–Tat fusion proteins to test for interactions of these factors with Tat. Western blot analysis of nuclear extract-derived proteins revealed selective binding of TFIIF, TFIIE and TFIIH (Fig. 2*a*), but not of TFIIB or TFIID (data not shown), to fusions with glutathione-S-transferase (GST) of Tat1, Tat2 or VP16, but not to GST alone. Involvement of the Tat and VP16 activation domains in these interactions was indicated by effects of the corresponding mutations (C22G and P18I in Tat, Δ 456/F442P in VP16) on TFIIH binding (Fig. 2*b*). The ability of a recombinant TFIIH subunit (p62) to bind to the Tat proteins with intact, but not mutated, activation domains argues for a direct interaction between Tat and TFIIH (Fig. 2*c*). A complementation assay demonstrated that the TFIIH that bound to Tat1, Tat2 or VP16 was transcriptionally functional (Fig. 2*d*). These data indicate direct or indirect interactions of Tat and VP16 with a TFIIH previously shown^{14–16} to play a role in phosphorylation of the Pol II CTD, which in turn could contribute to enhanced HIV-1 transcription elongation. This prediction agrees with observations that interactions of TFIIH with VP16 and Tat correlate with enhanced transcription elongation by these activators^{8,9}. In addition, because a Rev–VP16 fusion protein enhances elongation when targeted to a promoter-proximal RNA sequence in the HIV-1 promoter²², this result suggests that VP16 and Tat have similar effects upon transcription elongation.

As the TFIIH-associated kinase can phosphorylate the CTD within Pol II in a DNA-independent manner and in the context of a preinitiation complex (PIC)^{11,12}, we tested effects of Tat on TFIIH-mediated CTD phosphorylation in a simplified assay with a 90K GST–CTD fusion protein (Fig. 3*a*, lane 6) containing all 52 of the murine heptapeptide repeats²³. An affinity-purified TFIIH elicited a dose-dependent increase mainly in the hypophosphorylated form (GST–CTDa), and to a lesser extent in the hyperphosphorylated form (GST–CTDo) (Fig. 3*a*, lanes 1–5). At limiting

concentrations of either partially or highly purified TFIIH, Tat enhanced GST–CTD phosphorylation over 20-fold (Fig. 3*b*). The Tat activation domain (Tat1 1–48) was sufficient for this effect, and activation-defective mutations (C22G or P18I) in this region significantly reduced phosphorylation (Fig. 3*c*). Surprisingly, TFIIE, which can markedly stimulate TFIIH-mediated phosphorylation of the CTD in the context of Pol II (refs 11, 12), had no effect; this may reflect the need for an additional Pol II contact²⁴. Treatment of a partially purified TFIIH fraction with anti-p62 (TFIIH) antibodies, but not with antibodies against other general factors, reduced dramatically (>80%) the ability of this fraction both to phosphorylate the GST–CTD and to support either basal or Tat-stimulated HIV-1 transcription in a citrate-treated TFIIH-depleted extract (Fig. 3*d*). These results clearly demonstrate the ability of Tat to enhance CTD phosphorylation by TFIIH.

As TFIIH kinase activity efficiently supports hyperphosphorylation of the CTD in the context of Pol II (refs 11, 16) and as hyperphosphorylation of the GST–CTD substrate can occur with kinases different from the TFIIH-associated CAK kinase^{23,25}, it was surprising to see only hypophosphorylation of the CTD in our assay. However, a partially purified TFIIH fraction that also contains TFIIF did support hyperphosphorylation (Fig. 3*e*). The involvement of TFIIH in this reaction was demonstrated by the loss of hyperphosphorylation activity upon treatment with anti-p62 (TFIIH) antibodies and by equivalent DRB sensitivities (50% inhibition at 50 μ M) for the hyperphosphorylation and the purified TFIIH hypophosphorylation activities (data not shown). But as purified TFIIH and recombinant TFIIF could not recapitulate the hyperphosphorylation activity (data not shown), there must be an additional factor(s) in the preparation that helps to mediate this effect.

We found that citrate and H-8 kinase inhibitor were both potent inhibitors of dose-dependent TFIIH-mediated GST–CTD phosphorylation, by using either an anti-p62 (TFIIH)-purified TFIIH (Fig. 4*a*) or the partially purified hyperphosphorylating TFIIH (data not shown). The effect of citrate, but not of H-8, was substantially reversed by Tat, which parallels the ability of Tat to reverse citrate- but not H-8-inhibited HIV-1 transcription in extracts. This result supports the idea of a direct link between the effects of Tat on CTD phosphorylation and transcriptional

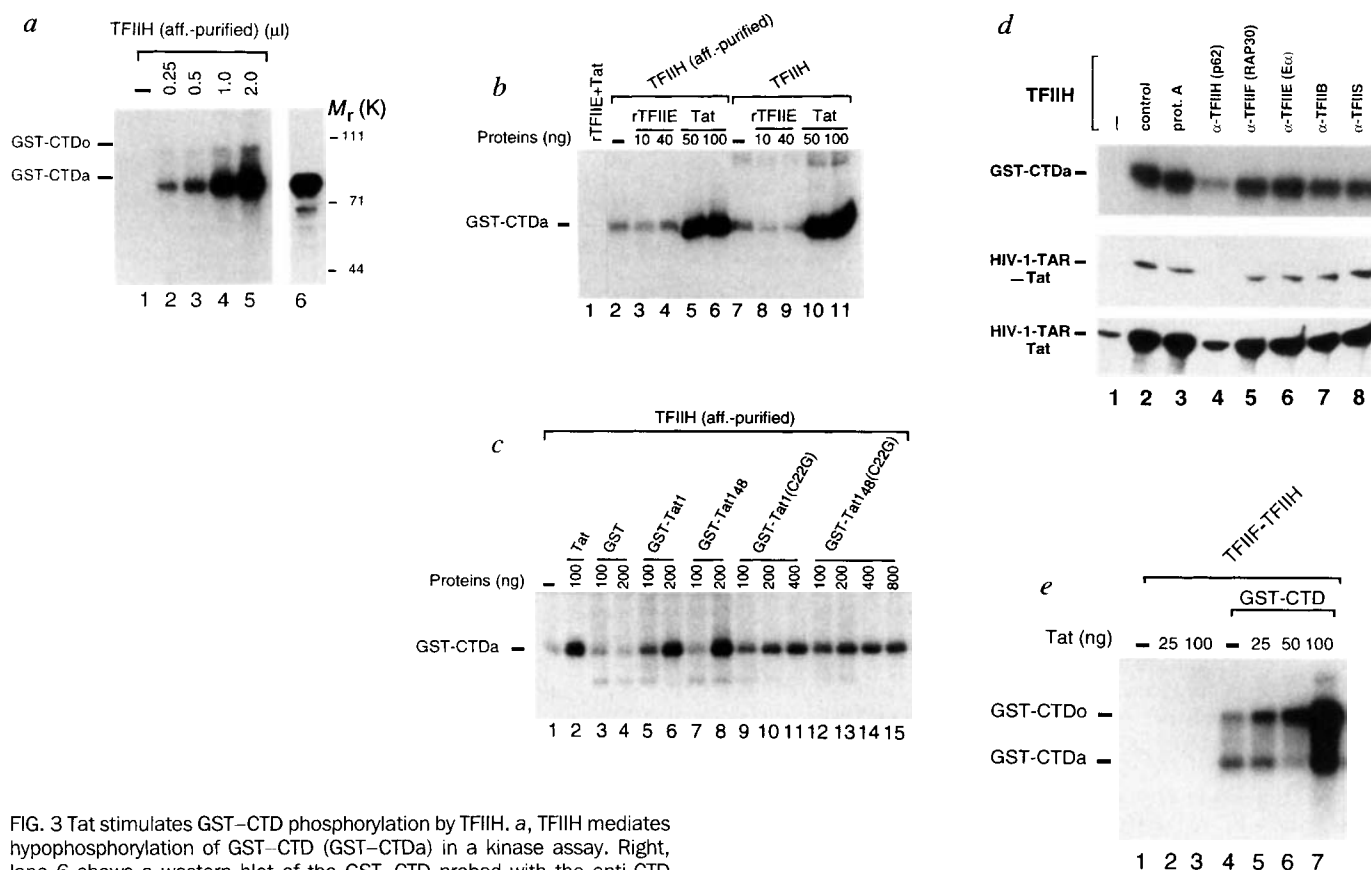


FIG. 3 Tat stimulates GST-CTD phosphorylation by TFIIH. **a**, TFIIH mediates hypophosphorylation of GST-CTD (GST-CTDa) in a kinase assay. Right, lane 6 shows a western blot of the GST-CTD probed with the anti-CTD monoclonal antibody 8WG16; on the right are shown the migration positions of relative molecular mass (M_r) markers in thousands. **b**, Tat, but not TFIIE, enhances hypophosphorylation of GST-CTD by TFIIH in the kinase assay. **c**, A mutation (C22G) in the activation domain of Tat decreases stimulation of GST-CTD phosphorylation by TFIIH. **d**, Specific depletion of TFIIH from a TFIIH fraction abolishes GST-CTD phosphorylation (top), as well as both basal and Tat-activated HIV-1 transcription (bottom). Kinase

reactions (with GST-CTD) and transcription reactions (with citrate-treated extract) were all performed with 2 µl of either untreated (control) or antibody-treated affinity-purified TFIIH fraction. **e**, Hyperphosphorylation of GST-CTD (GST-CTDo) by TFIIH is stimulated by Tat in the presence of other factors.

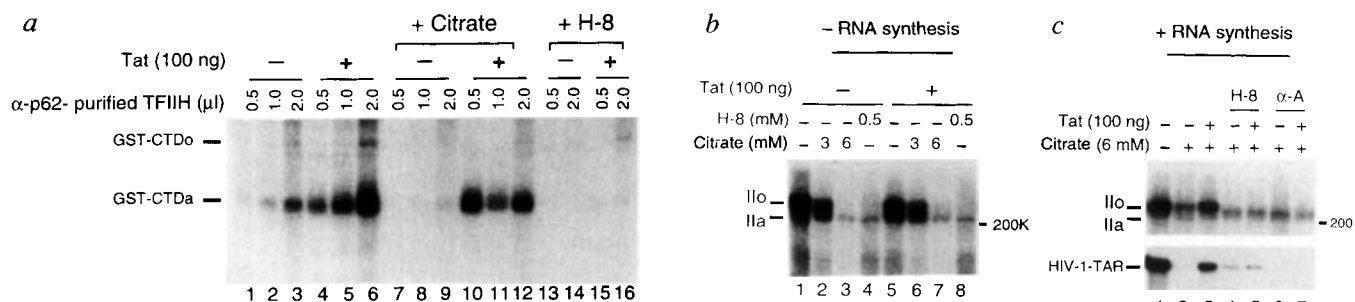


FIG. 4 Tat reverses citrate-mediated inhibition of CTD phosphorylation by TFIIH. **a**, Citrate inhibition of GST-CTD phosphorylation by TFIIH. GST-CTD was incubated with [γ - 32 P]ATP and an anti-p62-purified TFIIH fraction¹⁵, under the indicated conditions. **b**, Citrate inhibits Pol II CTD phosphorylation in a preinitiation complex. **c**, Tat overcomes citrate-mediated inhibition of Pol II CTD phosphorylation in the preinitiation complex in an RNA-synthesis-dependent manner. For **b** and **c**, Pol II CTD phosphorylation was carried out as described in Methods and 32 P-labelled phosphorylated Pol II was isolated using anti-CTD antibodies²⁶ and analysed by 7.5% SDS-PAGE. M_r markers are shown on the right in **b** and **c**. **d**, The CTD is required for efficient HIV-1 transcription elongation and for Tat function. Pol-II-depleted HeLa nuclear extract (with or without treatment with citrate or H-8) was complemented with either Pol IIA or Pol IIB in the presence of HIV-1 templates without and with Tat, as indicated.

enhancement. We therefore tested the effect of Tat on CTD phosphorylation of Pol II in transcription complexes, under conditions where HIV-1 transcriptional elongation is impaired by citrate or H-8. HIV-1 (pre)initiation complexes were formed in citrate- or H-8-treated extracts in the presence of dATP (to facilitate promoter melting) and [γ - 32 P]ATP (to radiolabel the CTD)²⁶ and with or without other ribonucleoside triphosphates (for RNA synthesis). Immunoprecipitation (using anti-CTD antibodies) and analysis of Pol II subunits showed that increasing levels of citrate or H-8 progressively inhibited formation of the hyperphosphorylated form (IIo) of the CTD-containing subunit (Fig. 4b, lanes 1–4). Consistent with the mechanism of action of Tat through nascent RNA binding^{1,4,5}, Tat markedly enhanced the formation of the hyperphosphorylated IIo subunit in the presence (Fig. 4c, top, lanes 1–3), but not in the absence (Fig. 4b, lanes 5–7), of RNA synthesis. CTD phosphorylation correlated precisely with the level of HIV-1 transcription (Fig. 4c, bottom). Under conditions of RNA synthesis, α -amanitin and H-8 both prevented IIo formation as well as Tat-enhanced HIV-1 transcription (Fig. 4c, lanes 4–7), confirming that these Tat functions are linked and require both nascent RNA synthesis and CTD kinase activity.

We also found that Tat function in an RNA polymerase-II-depleted, citrate-treated nuclear extract was partially restored by readdition of purified RNA polymerase IIA (containing the CTD), whereas addition of RNA polymerase IIB (lacking the CTD) only supported basal transcription to an extent comparable to that by RNA polymerase IIA in the presence of citrate or H-8 (Fig. 4d). These data indicate that the restriction imposed by citrate and H-8 is on CTD function and, further, they show that efficient HIV-1 transcription elongation in HeLa nuclear extract requires the CTD of Pol II, consistent with a CTD requirement for Tat function *in vivo*²⁷.

Our results reveal a new Tat function—stimulation of the activity of a TFIIF-associated kinase—and point to a key role for CTD phosphorylation in HIV-1 transcription elongation. Thus, interaction of Tat with TFIIF kinase may trigger phosphorylation of the CTD of Pol II under conditions in which TFIIF kinase cannot normally function, and so contribute to the formation of a processive elongation complex. Other cellular factors that may also contribute directly or indirectly to Tat function include the Tat-stimulatory factor²⁸ and both Tat and TAR RNA-binding proteins¹ and associated cellular cofactors^{29,30}. Given the complexity of the factors implicated in Tat function, and as several other kinases have the potential to phosphorylate the CTD of Pol II^{23,25}, activation of HIV-1 transcription by Tat may involve several factors acting at different steps. A cascade of phosphorylation events of the CTD by TFIIF and other kinases, or even phosphorylation–desphosphorylation cycles, could generate and maintain a highly processive HIV-1 elongation complex. This mechanism could also be used by other activators that alter processivity through TFIIF^{6,7,9}. To our knowledge, our study is the first demonstration that an activator can alter TFIIF CTD kinase activity in a manner that correlates with enhanced processivity. □

Methods

Transcription assays. HIV-1 transcription in nuclear extracts, either untreated or after treatment with citrate (6 mM) or H-8 (0.5 mM), was carried out as before^{5,17}. RNA products were isolated after RNase T1 treatment²⁸ and analysed on 7% polyacrylamide/50% urea gels. Recombinant Tat protein was prepared as described²⁷. HeLa nuclear extracts were depleted of either TFIIF or RNA Pol II by incubation, respectively, with either anti-p62(TFIIF) polyclonal or anti-CTD monoclonal (8WG16) antibodies in 400 mM KCl for 12 h at 4 °C.

Protein purification. The 4-column TFIIE/TFIIF/TFIIF (Mono-S fraction) the 5-column TFIIF/TFIIF (HPLC-SP fraction) and the 7-column TFIIF (SP-5PW fraction) were isolated as described^{12,14,16}. Affinity(GST–VP16)-purified TFIIF was prepared as described⁸. Pol IIA and Pol IIB were purified from HeLa nuclear pellets (E. Martinez and R.G.R., unpublished) and the relative amounts of Pol IIA and Pol IIB were determined by western blot analysis with antisera against the 140K subunit of Pol II. The presence or absence of the CTD in each Pol II preparation was established by immunoblot analysis with anti-CTD antibodies and by CTD phosphorylation using TFIIF.

Protein–protein interaction assays. 30 μ l of 10 mg ml^{−1} HeLa nuclear extract (Fig. 2a, b) or 50 ng recombinant p62 protein (Fig. 1c) were mixed with 2 μ g GST or GST fusion proteins immobilized on glutathione–Sepharose beads and the mixture brought to 100 μ l with buffer BC (50 mM Tris-HCl, pH 7.9 at 4 °C, 20% glycerol, 0.2 mM EDTA, 0.5 mM PMSF and 2 mM DTT) containing 100 mM KCl and 0.3% NP-40. After incubation for 4 h (extract) or 2 h (p62) at 4 °C, resin-bound complexes were washed with BC buffer containing 150 mM NaCl and 0.5% NP-40, and bound proteins were analysed on 10% SDS–PAGE followed by western blotting. Affinity-purified fractions were obtained by mixing 600 μ l of 10 mg ml^{−1} HeLa nuclear extract with 20 μ g of immobilized GST or GST fusion proteins. After 4 h at 4 °C, bound fractions were washed as before, eluted with 100 μ l of buffer BC containing 1 M KCl, dialysed to 100 mM KCl, and tested in transcription. Recombinant p62 was obtained as described²⁴. Wild-type and mutant Tat–GST fusion proteins have been described²⁵.

GST–CTD phosphorylation assay. Reactions (15 μ l) were done in kinase buffer (KB) containing 50 mM Tris-HCl (pH 7.5), 5 mM MnCl₂, 40 mM KCl, 2% glycerol, 0.25 mg ml^{−1} BSA, 1 mM DTT and 0.3% NP-40. Protein components were mixed at 23 °C and 2 μ M ATP plus 10 μ Ci [γ - 32 P]ATP was added. Unless otherwise noted, amounts of the protein components, when present, were: GST–CTD, 100 ng; affinity-purified TFIIF, 0.25 μ l; purified TFIIF, 0.125 μ l; partially purified TFIIF–TFIIF, 0.125 μ l; Tat, 100 ng; and TFIIE, 20 ng. After incubation at 30 °C for 60 min, immobilized phosphorylated GST–CTD protein was washed with 50 mM Tris-HCl (pH 7.9), 5% glycerol, 1 mM EDTA, 150 mM NaCl and 0.5% NP-40, and analysed on 10% SDS–PAGE. GST–CTD protein was purified as described²³, bound to glutathione–Sepharose beads and stored at −70 °C. TFIIF depletion involved mixing 5 μ l affinity-purified TFIIF fraction with protein-A-bound antibodies (Fig. 3e) for 1 h on ice. The mixture was pelleted briefly and the supernatant tested in kinase and transcription assays. Recombinant TFIIE was prepared as described²⁴.

Pol II CTD phosphorylation in (pre)initiation complexes. For Fig. 4b, preinitiation complexes were formed by incubating (for 20 min at 30 °C) the HIV-1-TAR-G400 template with HeLa nuclear extracts, with or without pretreatment with citrate or H-8, in transcription buffer¹⁷ lacking nucleoside triphosphates. Protein phosphorylation was monitored by subsequent incubation (30 min, 30 °C) with 20 μ M dATP, 0.5 μ M ATP and 80 μ Ci [γ - 32 P]ATP in the presence or absence of Tat. For Fig. 4c, after formation of preinitiation complexes in HeLa nuclear extracts without or with citrate or H-8 (500 μ M) treatment (Fig. 4b), Tat and α -amanitin (2 μ g ml^{−1}) were added to the reaction mixtures. Simultaneous protein phosphorylation and transcription were carried out for 30 min at 30 °C after addition of 20 μ M dATP, 200 μ M each of CTP, GTP and UTP, 0.5 μ M ATP, and either 80 μ Ci [γ - 32 P]ATP (to monitor CTD phosphorylation) or 12.5 μ M [α - 32 P]UTP (to monitor transcription).

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Crystal structure of a DExx box DNA helicase

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THERE are a wide variety of helicases that unwind helical DNA¹ and RNA substrates². The twelve helicases that have been identified in *Escherichia coli*¹ play a role in almost all cellular processes involving nucleic acids. We have solved the crystal structure of a monomeric form of a DNA helicase from *Bacillus stearothermophilus*, alone and in a complex with ADP, at 2.5 and 2.9 Å resolution, respectively. The enzyme comprises two domains with a deep cleft running between them. The ATP-binding site, which is situated at the bottom of this cleft, is formed by motifs that are conserved across the superfamily of related helicases. Unexpected structural homology with the DNA recombination protein, RecA, suggests how ATP binding and hydrolysis may drive conformational changes of the enzyme during catalysis, and implies that there is a common mechanism for all helicases.

Mechanistically, there are two classes of helicase: those that can displace a polynucleotide strand from its 3' end, and those that displace a strand from the 5' end¹. Although the enzymes share sequence homology within these classes, there is little sequence homology between them. Furthermore, some helicases are hexameric (reviewed in ref. 3), whereas others are dimeric^{4,5} or form a part of larger multiprotein complexes⁶. At first, it seems difficult to understand how this range of oligomeric structures, activities, specificities and sequences might be accommodated within a single molecular structure. The helicase we have studied is related most closely to PcrA of *Staphylococcus aureus*⁷ (62% sequence identity), but also shares significant homology with *E. coli* Rep¹ and UvrD⁵ (41% and 44% identity, respectively). In common with Rep and UvrD, it can unwind DNA duplexes from a free 3' end (unpublished data). PcrA contains all of the signature helicase motifs⁸ and, consequently, is also related to the vast number of RNA helicases that have been identified in eukaryotes².

Details of the structure determination and refinement are presented in Table 1. The enzyme comprises two domains: domain 1 consists of residues 1–286, domain 2 residues 287–650 (Fig. 1). Both domains contain two subdomains that we will refer to as A and B. Each domain has a subdomain (1A and 2A) comprising a central parallel β -sheet flanked by α -helices. These subdomains have homology with each other, but also with the central region of the RecA protein⁹. Both 1A and 2A have large insertions, at equivalent positions, which then go on to form subdomains 1B and 2B. Each of these subdomains is α -helical, but they are structurally different. Overall, the molecule is flattened with a shape reminiscent of a crab claw with one pincer larger than the other. The two α -helical domains are in contact and close off a cavity that runs through the centre of the enzyme. Although the diameter of this cavity is insufficient to accommodate a DNA duplex in this structure, it is likely that the cleft is involved in recognition of the DNA substrate (see below).

The bound ADP was located in a pocket at the base

of the cleft between the domains of the protein (Fig. 2). Sequence alignments of 3'–5' DNA and RNA helicases⁸ have revealed a series of conserved motifs (Fig. 2a), most of which are located around the ATP-binding site (Fig. 2b). The crystal structure also suggests roles for these motifs in the catalytic mechanism. Motif I contains the 'Walker A' motif¹⁰ and is responsible for binding the triphosphate tail of the ATP¹¹. Motif Ia forms the edge of a shallow groove across the surface of the protein, and may be involved in binding to DNA. Motif II is the 'DEAD' box motif², and has been implicated in the binding of Mg²⁺, which is required for hydrolysis of ATP in both RNA^{11,12} and DNA helicases¹³, although there is no metal ion bound in our structure. Mutational analysis of motif III in the RNA helicase, eIF-4A¹¹, indicates that this motif is involved in the coupling of ATP hydrolysis to helicase activity. Motif IV forms the linkage between the two domains of the protein, running underneath and then forming a part of the ATP-binding site. *B. stearothermophilus* PcrA can use a wide variety of nucleotides including ATP, dATP, GTP, dGTP and CTP (L.E.B. *et al.*, unpublished data). This lack of specificity for the nucleotide co-factor can be explained by the nature of the interactions with the enzyme, as there are very few specific interactions with the base (in particular with the 6-amino group of the adenine), simply a stacking interaction with a conserved tyrosine side chain from motif IV. Furthermore, there are no interactions between the hydroxyls of the sugar moiety and the enzyme. Motifs V and VI contribute both to the sides of the ATP-binding site and the domain interface, and may be important for the helicase activity of the protein.

The location of the ATP-binding site is likely to be very important for the mechanism of the enzyme because it is situated

TABLE 1 Structure determination

Data collection						
Data	λ (Å)	Resolution (Å)	R_{sym} (%)	Completeness* (%)		
Native	0.87	2.5	4.9	96.6 (98.8)		
Mercury	1.54	3.1	9.3	91.9		
Lead	1.54	3.1	9.8	93.8		
Gold	1.54	3.1	9.7	95.5		
Platinum	1.54	3.2	9.8	95.1		
Iridium	1.54	3.2	9.5	92.9		
Selenomethionine	1.54	3.2	10.4	91.6		
ADP soak	1.54	2.9	8.8	97.1 (97.4)		
Phasing statistics						
Derivative†	Anomalous data	No. of sites	MFID	R_{Cullis}	Phasing power	Mean f.o.m.
Thimerosal (10 mM, 60 h)	yes	2	0.14	0.79	0.8	0.60
Pb acetate (100 mM, 24 h)	yes	5	0.15	0.73	1.0	
NaAuCl ₄ (10 mM, 72 h)	yes	5	0.21	0.83	0.9	
K ₂ PtCl ₄ (2 mM, 72 h)	yes	7	0.33	0.84	0.9	
K ₂ IrCl ₆ (10 mM, 48 h)	yes	6	0.21	0.83	1.0	
Selenomethionine	no	16	0.13	0.71	0.9	
Refinement						
	Native		ADP complex			
Resolution (Å)	2.5		2.9			
Final <i>R</i> -factor (all data, 10 Å to resolution limit)	21.5		20.6			
<i>R</i> _{free} (5% of data)	26.9		26.8			
R.m.s.d. bonds (Å)	0.012		0.013			
R.m.s.d. angles (deg)	2.96		3.00			

* Numbers in parentheses indicate completeness in highest-resolution shell.

† Conditions for derivative preparation are shown in parentheses.

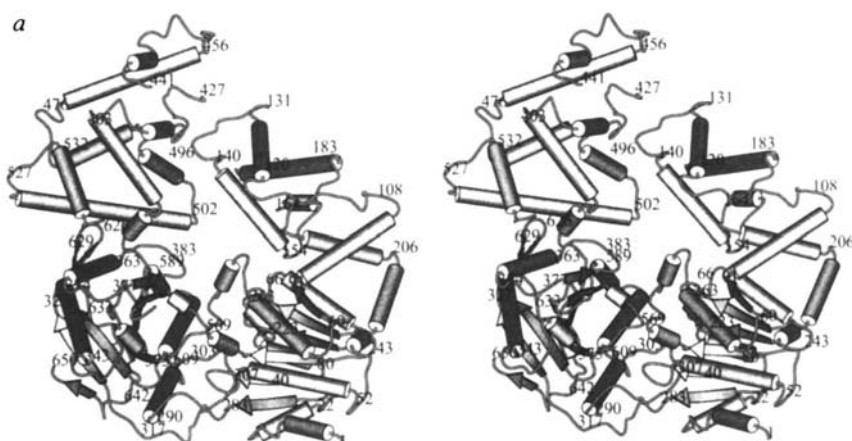


FIG. 1 a, Stereo view of the overall fold of the protein (prepared using the program PREPPI). b, The domain structure of the protein. Domain 1A is shown in green, domain 1B in yellow, domain 2A in red, and domain 2B in blue (figure generated using RIBBONS).



ideally to affect the relative positions of the two large domains in response to the binding and/or hydrolysis of ATP. Such conformational changes in response to ATP binding and hydrolysis have an important role in the mechanism of these enzymes. Mutation at several different positions within the conserved motifs have been shown to affect the ATPase activity of a variety of helicases^{11,12,14,15}, but even more important, from a mechanistic viewpoint, are those mutations resulting in uncoupling of ATPase and helicase activities^{11,12}. Several of these residues are in contact with each other across the domain interface, suggesting that such contacts may be important for coupling the helicase activity to the binding and hydrolysis of nucleotides.

The similarity between the structures of the A domains and that of the RecA protein^{9,16} is striking (Fig. 3a). We therefore determined the structure of a complex with ADP to compare it with the RecA/ADP complex¹⁶. This comparison reveals that all of the key residues in the active site of RecA are conserved in space in the helicase active site (Fig. 3b), although their positions in the linear sequences are different. The conservation of residues in the active sites of these proteins implies that they have similar roles in the catalytic mechanisms. Although the triphosphate-binding site is

	Motif I (31 - 40)	Motif Ia (61 - 72)	Motif II (223 - 229)	Motif III (249 - 262)
<i>E. coli</i> Rep	AGAGSGKTRV	AVTFTNKAAREM	DEYQDTN	VGDDQSIYSWRGA
<i>E. coli</i> UvrD	AGAGSGKTRV	AVTFTNKAAREM	DEFQDTN	VGDDQSIYGWARGA
<i>S. A.</i> PcrA	AGAGSGKTRV	AITFTNKAAREM	DEYQDTN	VGDSQSIYGWARGA
<i>B. S.</i> PcrA	AGAGSGKTRV	AITFTNKAAREM	DEYQDTN	VGDADQSIYRWARGA

	Motif IV (280 - 288)	Motif V (562 - 575)	Motif VI (599 - 611)
<i>E. coli</i> Rep	IKLEQNYRS	MTLHASKGLEFPYV	EERLAYVGITRA
<i>E. coli</i> UvrD	IRLEQNYRS	MTLHSAKGLEFPQV	EERLAYVGVTRA
<i>S. A.</i> PcrA	IFLEQNYRS	MTMHSKGLEFPYV	EERRICYVAITRA
<i>B. S.</i> PcrA	ILLEQNYRS	MTLHAASKGLEFPYV	EERLAYVGVITRA

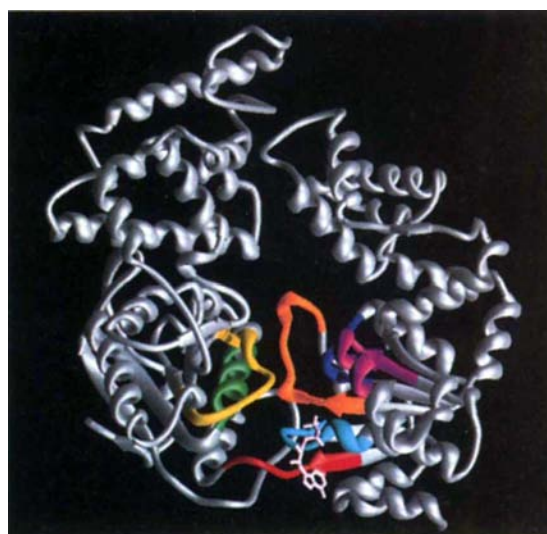
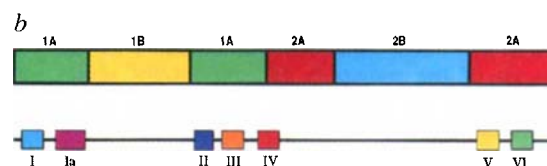


FIG. 2 a, Sequences of the conserved motifs in Rep and UvrD from *E. coli*, PcrA from *S. aureus* (*S. A.* PcrA) and PcrA in *B. stearothermophilus* (*B. S.* PcrA). Numbers refer to the *B. stearothermophilus* PcrA sequence. b, The relationship between the linear sequence and the position of the conserved helicase motifs and their positions within the helicase domain structure. The colour scheme for the domains is the same as that in Fig. 1b. The bound ADP is depicted in pale lilac.

conserved, the conformation of the remainder of the nucleotide co-factor is very different to RecA, being rather more closely related to other NTPases which share the overall architecture of the active site but have a different connectivity¹⁶.

The similarity between the structures of RecA and domains 1A and 2A of the helicase suggests that the enzymes may share aspects of their biochemical mechanisms. Both proteins undergo conformational changes in response to binding and hydrolysis of ATP. It has been proposed that the magnesium ion binding site in RecA is contributed by an aspartate (Asp 144)¹⁶ which corresponds to Asp 223 in motif II in the helicases. Mutational analysis of residues in this motif support a similar role in the helicases¹¹⁻¹³. The side chain of Glu 96 in RecA is in the same position in space as Glu 224 of motif II in PcrA, although these residues originate from different parts of the structures. In RecA, this residue is thought to activate the attacking water during the hydrolysis of ATP¹⁶. A glutamine residue is conserved in the structures of both enzymes (Gln 194 in RecA and Gln 254 in PcrA) (Fig. 3b). This residue has been proposed to propagate conformational changes in RecA, resulting from the binding of ATP, which affect the DNA-binding properties of the protein¹⁶. The DNA-binding site in RecA is thought to involve two loops of polypeptide that are disordered in the RecA structure⁹. These loops correspond to residues 227-232 and 254-264 in the helicase structure and are adjacent in sequence to motifs II and III, respectively (Fig. 4). These two

regions of the structure are located at the bottom of the cleft between the two domains and at one end of the shallow, positively charged groove across the surface of the protein. Consequently, the binding and/or hydrolysis of ATP could be transmitted across the protein and affect the affinity of the enzyme for double-stranded or single-stranded DNA. It has been shown that this alternation in affinity for different forms of DNA is the basis for the mechanism of Rep helicase¹⁷. Furthermore, because RecA binds single-stranded DNA with a defined directionality¹⁸, the comparison with the proposed single-stranded DNA-binding site in the helicase structure suggests that the directionality of the bound DNA will be the same. This orientation is consistent with a model in which a region of double-stranded DNA binds in the central cleft, and the 3' DNA tail, which is produced by the helicase reaction, lies across the top of the RecA-like domain 1A and the groove across the surface of the protein. The program GRASP (A. Nicholls and B. J. Honig) was used to calculate the surface potential for this helicase structure (Fig. 5). These calculations show that the surface potential of the enzyme is highly negative over most of the molecule, with the exception of the central cavity and the shallow groove across one face, suggesting that either or both of these regions could be involved in DNA binding. If a DNA duplex were to bind in the central cavity, then a conformational change would be required to allow access of this site to DNA. This conformational change could be linked to hydrolysis or binding of ATP. It

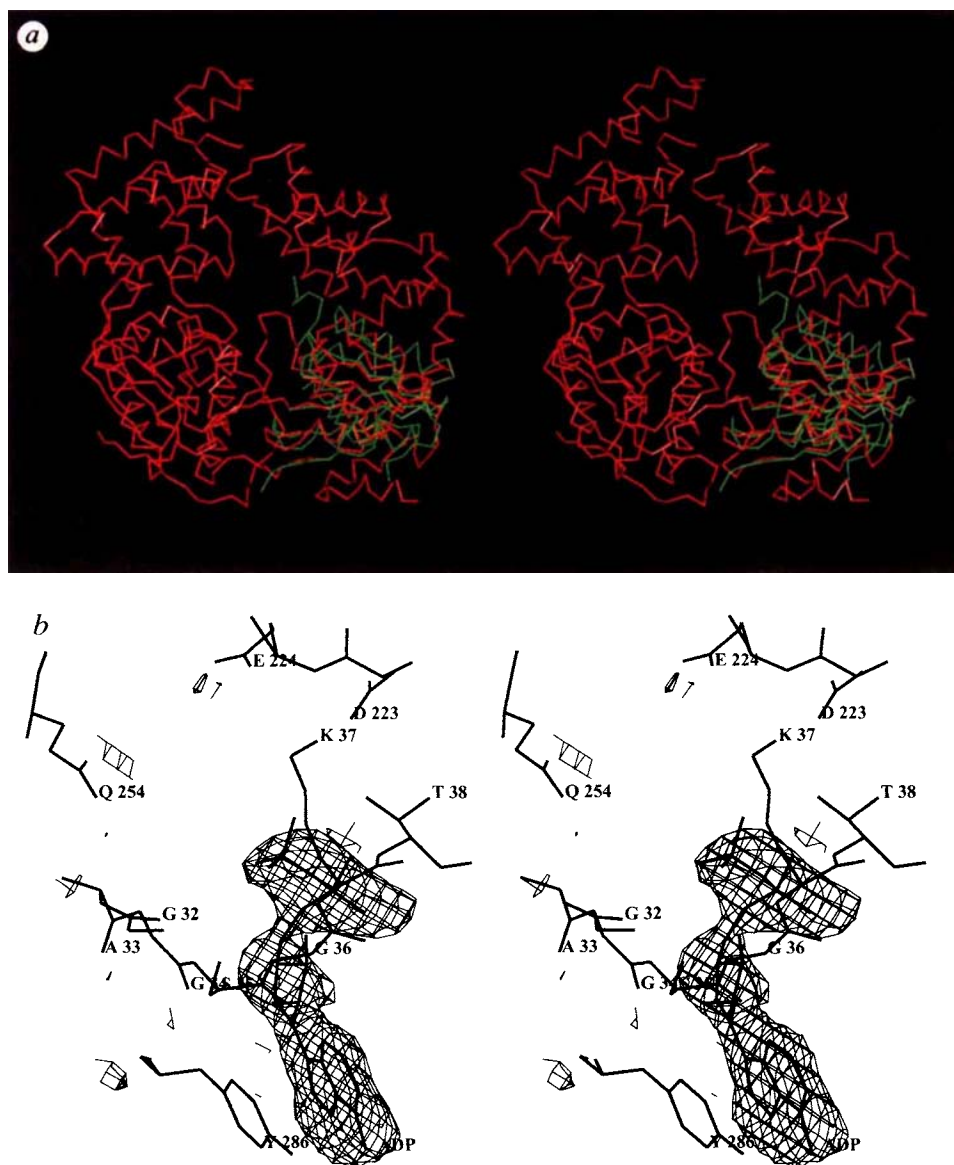


FIG. 3 a, Stereo figure showing the comparison of the helicase structure (in red) with that of the ATPase domain of RecA (green). b, Stereo view of the ADP-binding site of PcrA. The $F_o - F_c$ difference electron density, calculated before ADP was included in the model, is contoured at a level of 2.5σ .



FIG. 4 Diagram illustrating how the ADP-binding site and the proposed DNA-binding site are connected. The bound ADP is shown in magenta, and the loops associated with motifs II and III are in red and blue, respectively. The side chains of residues Asp 223 and Glu 224 are shown in red, and Glu 254 in blue.

has been shown¹⁷ previously that the affinity of the Rep helicase for single-stranded and double-stranded DNA is highly dependent upon ATP binding and hydrolysis.

The apparently disparate nature of helicase enzymes with regard to sequence, activity, oligomeric assembly and substrate specificity at first suggests that the enzymes are unlikely to share a common fold. Although the structures of individual enzymes will vary in detail, we propose that the enzymes actually have more in common than might at first be apparent. The unexpected similarity between domains 1A and 2A of PcrA, and their similarity with RecA, suggests a common basis for the mechanism of RecA and the 3'-5' helicases. Furthermore, the conserved motifs are found not only in the sequences of helicase with known 3'-5' activity, but also in some enzymes with 5'-3' specificity^{19,20}, indicating that both specificities can be associated with the same basic structure. Furthermore, it has been shown that Rho, a hexameric 5'-3' RNA/DNA helicase, shares sequence homology not only with DNA helicases but also with F₁ ATPase²¹, and in the latter case the homology extends across a much larger region. This has led to the proposal that the ATP-binding domain of Rho has a structure similar to those known for the ATP-binding domains of F₁ ATPase²² that share the same fold as RecA⁹. These similarities are particularly significant as the F₁ ATPase α and β subunits are arranged in a pseudo-six-fold arrangement which is highly reminiscent of images in hexameric helicases such as Rho, RuvB, T7

gene 4 protein and DnaB obtained by electron microscopy (reviewed in ref. 3). A model for the structure of Rho helicase has been proposed on the basis of these comparisons coupled with data from mutant enzymes²³. The structure of PcrA extends this argument and indicates that all helicases contain RecA-like domains. Studies on a hexameric form of RecA (ref. 24, and E. Egelman, personal communication) indicate considerable similarities in structure with the hexameric helicases. It is therefore likely that there are features of the mechanisms which are common to RecA and both classes of helicase. All of the proteins require signalling between different regions of the proteins which link ATP binding and hydrolysis to conformational changes in the proteins and in turn affect the affinity of the enzyme for different forms of DNA, thereby driving a biochemical reaction. In the case of RecA, these events presumably begin with the destabilization of a DNA duplex to allow the formation of a DNA triplex, although for the helicases only the first part of this reaction is catalysed. Our comparisons of the structures of RecA, F₁ ATPase and PcrA show a conservation of function for residues in and around the ATP-binding sites, and also suggest how binding and hydrolysis of ATP is signalled to the DNA binding sites on the proteins; this suggests a common basis for the coupling of ATP binding/hydrolysis to the conformational changes in the proteins that are at the heart of their catalytic mechanisms. It has been shown that the binding of ATP is highly cooperative in helicases^{17,25,26}. One consequence of this cooperativity is that the hexameric helicases exhibit 'half-sites' reactivity, with three high-affinity binding sites for ATP and three sites of low or unmeasurable affinity. In PcrA, although domains 1A and 2A share similar folds, only 1A can bind ATP, owing to replacement of the residues around the ATP-binding site in domain 2A. This is a convenient way to ensure 'half-sites' reactivity of a protein with two potential ATP-binding sites and, given the analogy with the hexameric helicases, may well be important in the overall mechanism of the enzymes.

There are presently over 250 proteins in the sequence databases that carry some or all of the 'signature' motifs of helicases⁸. It is evident from sequence analyses that most of these enzymes are likely to retain the two RecA-like domains. The internal repeat of the structure suggests strongly that these helicases may have evolved from a gene-duplication event, after which the two domains diverged in structure and function to generate enzymes with different activities and substrate specificities. Furthermore, several helicases are deleted in regions corresponding to domains 1B and 2B or show the greatest variation in these regions⁸. This suggests that the minimal structure required for helicase activity *per se* is the two conserved RecA-like domains, but that other regions may be involved in polynucleotide substrate binding and recognition, or for other biochemical activities. This is exemplified by the primosomal protein PriA. Sequence analysis⁸ and biochemical data²⁷, when combined with the structure presented here, suggest that domain 1B is missing and that domain 2B has been replaced by a zinc-finger motif. PriA is a 3'-5' helicase that shows a specificity for the primosome assembly site on DNA²⁸. Disruption

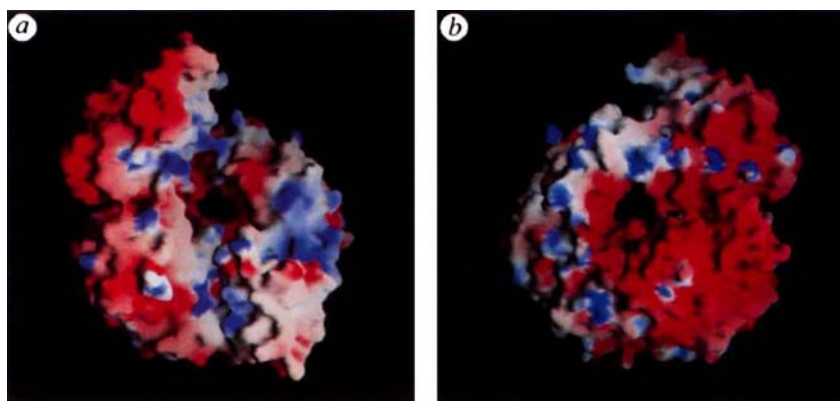


FIG. 5 Electrostatic surface of both sides of the protein (a and b), with a being the same view as Fig. 1b. Negative potential is shown in red, positive potential in blue (prepared using the program GRASP).

of ATPase activity has no effect on assembly of the primosomal complex²⁹, demonstrating a structural role for the protein that is independent of its helicase activity.

The structures presented here provide an explanation for the conservation of the seven helicase signature motifs, and the unexpected similarity of the structure to that of RecA provides evidence for a common mechanism for DNA helicases. The structures also indicate how the basic helicase function might be adapted in different proteins to accept different substrates or to alter their activity. However, further structural information, particularly a structure of a hexameric helicase, will be required to obtain a more detailed understanding of the mechanism of these enzymes. □

Methods

Cloning, purification, characterization and crystallization will be described elsewhere. The crystals belong to the space group $P6_3$, with unit cell dimensions $a = 138.5 \text{ \AA}$, $c = 111.1 \text{ \AA}$ and a solvent content of 65%. Integrated intensities were calculated and scaled using the programs DENZO and SCALEPACK (Z.

Otwinowski & W. Minor). The mercury sites in the thimerosal derivative were determined by difference Patterson and Fourier syntheses. The heavy-atom sites in other data sets were determined by difference Fourier calculations. A $3.1 \text{ \AA}$ map was calculated using the MIR phases. The CCP4 (Daresbury) program suite was used unless otherwise stated. The initial map was solvent flattened using the program DM (K. Cowtan), assuming a solvent content of 60%. An initial model was built into the solvent-flattened map using the graphics program TURBO FRODO (A. Roussel and C. Cambillau). The phases were improved further by combination with phases derived from the model, followed by solvent flattening. The model was refined initially using positional refinement in XPLOR (A. Brünger, M. Karplus and G. Petsko), followed by simulated annealing refinement. Manual rebuilding was performed between the refinement cycles. The crystallographic R_{free} (ref. 30) was monitored at each stage to prevent model bias. The C-terminal 74 residues of the protein are disordered, as are a few residues in a couple of surface loops. In light of the large region of disorder at the C terminus, we measured the relative molecular mass (M_r) of the protein by electrospray mass spectrometry (data not shown). An M_r of $82,494 \pm 14$ was obtained, in good agreement with the predicted weight of 82,435 (unpublished data) for the full-length protein. Crystals were collected in 1.2 M sodium acetate, 100 mM MES, pH 6.4. Heavy-atom derivatives were prepared by transferring the crystals into this solution containing heavy-atom compounds. The ADP complex was obtained by transferring the crystals to a buffer of lower ionic strength (100 mM NaCl, 10 mM MgCl_2 , 10 mM MES, pH 6.4) containing 5 mM ADP. Crystals were soaked in this solution for 4 h before data collection.

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CORRESPONDENCE and requests for materials should be addressed to D.B.W. (e-mail: wigley@biop.ox.ac.uk). Coordinates have been deposited at the Brookhaven Protein Data Bank (accession code 1PJR).

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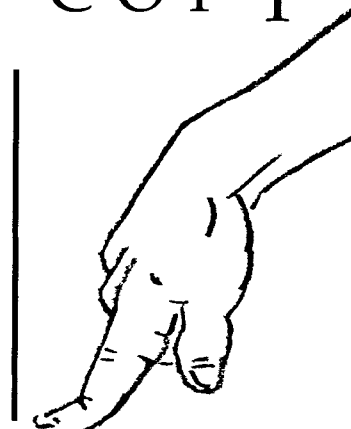
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A human RNA polymerase II complex associated with SRB and DNA-repair proteins

Edio Maldonado, Ramin Shiekhatter, Michael Sheldon, Helen Cho, Ronny Drapkin, Paula Rickert, Emma Lees, Carl W. Anderson, Stuart Linn & Danny Reinberg

Nature 381, 86–89 (1996)

FIGURE 1 is reproduced again here because the original image reproduction was incorrect and masked critical bands in the gels. □

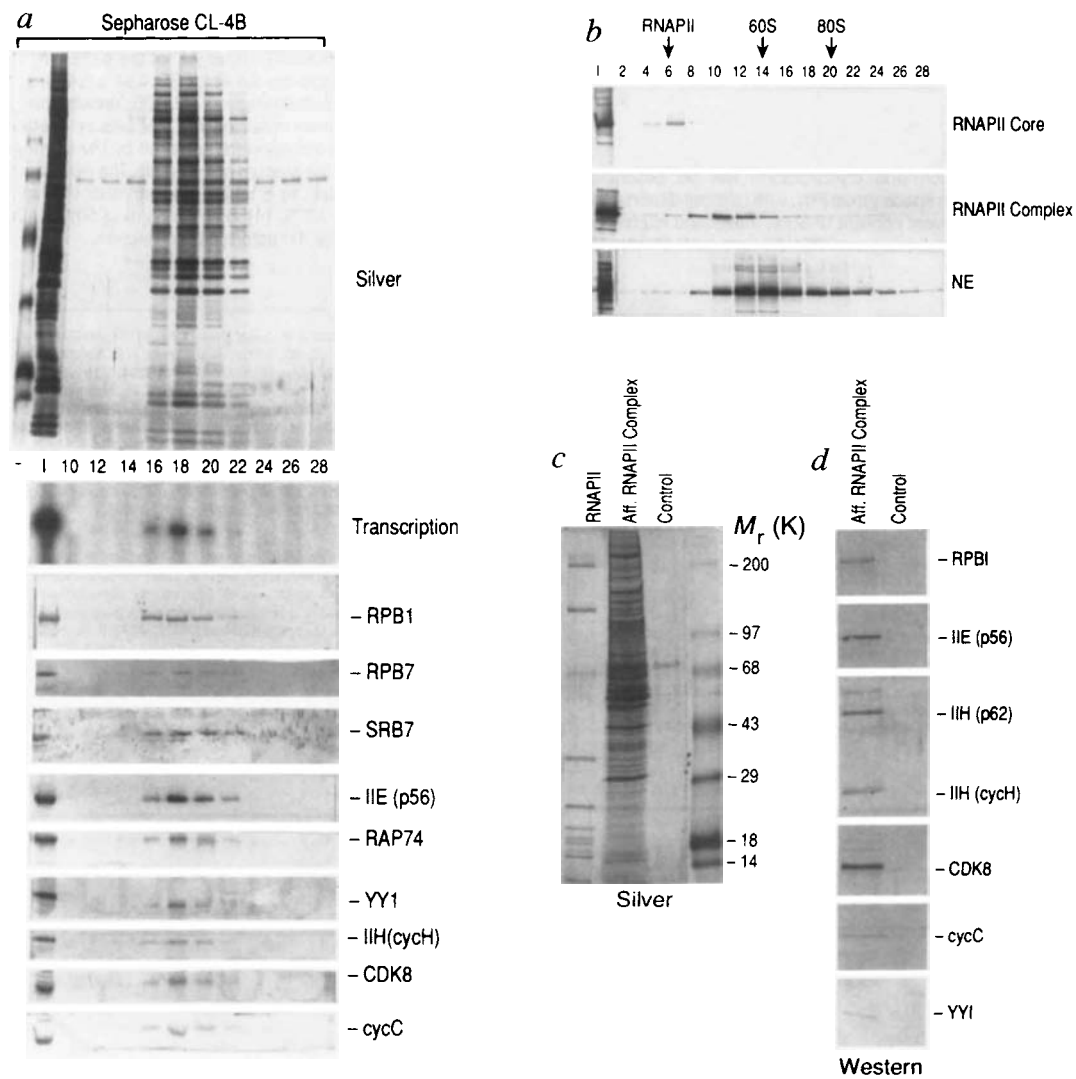


FIG. 1

New twists on DNA technology

This week Product Review covers items such as reagents for cell detachment and tissue dissociation, reverse transcription and liposomal transfection, as well as custom-synthesized oligonucleotide tubes and microplates.

UTILIZING a new positive selection system to detect *in vivo* DNA mutations, the MutaPlax cII-Select kit from Epicentre Technologies is designed to give highly accurate mutation frequency data for mutagenesis assays using rodents or cell lines transgenic for bacteriophage lambda. Mutations are detected by direct selection, avoiding the need to screen large numbers of plaques and reducing the number of false positives caused by host cell-derived mutations. Expensive specialized media, reagents and accessories for screening are not required, as mutation selection is performed using only standard-size Petri dishes and standard growth agar.

Reader Enquiry No. 100

Molecular Grinding Resin from Geno Technology has been developed specifically for the effective **grinding of biological** samples for the extraction of DNA, RNA and proteins. The resin is composed of high-tensile microparticles, which effectively disrupt nuclei and other cell organelles. Moreover, the resin does not bind proteins or nucleic acids: simply mix the biological samples with the resin and grind or homogenize the sample. Unlike sand or other grinding materials, this resin



Geno Technology's cellular grinding resin.

does not damage genomic DNA or high molecular mass proteins, according to the manufacturer. The grinding resin can be used with any homogenization technique, including high-speed mechanical grinders such as Polytron and high-energy sonicators. It is RNase- and DNase-free and eliminates the need to use liquid nitrogen for many applications.

Reader Enquiry No. 101

Techne now offers its Genius **thermal cycler**, designed for the high throughput of samples. Seven units are available, all with a heated lid as standard. (The heated lid is required to reduce refluxing of the sample onto the lid and side walls of the sample tube.) Models are available to suit 0.5-ml



Techne's high-throughput thermal cycler.

microcentrifuge tubes, 0.2-ml microtubes, 96-well microplates, 384-well microplates and microscope slides. Interchangeable blocks have been designed in such a way that a block can be changed in a matter of seconds without the need for any tools. Genius operates over a temperature range from 4 °C to 99 °C, with a stated block uniformity of ± 0.5 °C, as well as incremental and decremental temperature and time control. Other features include programme-to-programme linking, a maximum number of repeat cycles per programme of 99, a pause button and programmable ramp rates all as standard. Additional features are also available using the optional RS232 software package.

Reader Enquiry No. 102

Innovative Cell Technologies has introduced Accutase, a new reagent that has been designed to perform **enzymatic cell detachment and tissue dissociation**. This product should help maximize cell detachment, as well as viability of a wide range of cell types, including fibroblasts, vascular

endothelial cells, hepatocytes, CHO and BHK cells, and adherent tumour cell lines. Accutase contains non-mammalian proteolytic and collagenolytic enzymes, and has been shown to be effective in tissue dissociation of embryonic tissues for isolation of neurons and hepatocytes, as well as and in bone marrow cell isolation. Accutase is available in a ready-to-use 100-ml 1× solution or in a 10× concentrate for tissue dissociation protocol development. Cells typically detach in a stated 5–10 min and can be harvested and replated as usual; protease inhibitors are not required.

Reader Enquiry No. 103

cDNA synthesis

Boehringer Mannheim's Expand RT has been formulated to be a high-performance **reverse transcriptase**. The genetically engineered enzyme offers up to a stated fivefold increase in yield and cost savings of 75 per cent. It is also said to offer higher processivity, with synthesis of cDNA fragments up to 13.5 kb in length. These characteristics should make it a useful tool for copying longer RNAs, the synthesis and amplification of full-length cDNAs and maximal conversion of rare mRNA into cDNA, the company explains. The main applications include RT-PCR, the generation of full-length cDNA libraries and the detection of rare mRNAs. It may also produce improved results in many RT-PCR applications, such as 5'–3' RACE, as well as the study of mRNA expression and regulation elements and the investigation of RNA viruses.

Reader Enquiry No. 104

Amersham has launched a new **first strand synthesis kit for RT-PCR**. The new kit can be used to produce full-length cDNA from low levels of mRNA. It generates cDNA that can be used directly in RT-PCR experiments. Each kit is supplied with protocols and sufficient reagents to perform 100 reactions starting with as little as 1 µg of purified mRNA or 5 µg of total RNA. Two types of primer are supplied with the kit: anchored oligonucleotide dT₂₅ primers and random hexamers, although specific oligonucleotide primers can also be used. This choice means the most appropriate primer can be chosen depending on the nature of the target mRNA. Anchored oligonucleotide dT₂₅ primers ensure that

the resulting cDNA contains the minimum sequence from the poly(A) tail of the message, increasing the potential for full-length synthesis. The kit can be used with either purified mRNA or with total RNA if the target sequence is of medium-to-high abundance. Included are control mRNA and PCR primers, which can be used to test the first strand cDNA reaction and the subsequent PCR amplification step.

Reader Enquiry No. 105

DNA separation

Resolver Gold from R&D Systems is designed to be a rapid and easy method to **separate co-migrating DNA samples**. Addition of this product to agarose gels is said to allow the separation of DNA fragments that normally co-migrate, but have different base compositions. This is a DNA intercalating molecule that is covalently



Resolver Gold from R&D Systems.

bound to a large polymer. Once it has intercalated within the DNA, the attached polymer increases friction of a given molecule and hence slows its migration through an agarose gel. Intercalation occurs preferentially within A+T rich sequence motifs of DNA. Now, a mixture of DNA that would normally co-migrate can be separated owing to their differing sequences. The manufacturer states that sequences with as little as one per cent difference in A+T content may be resolved using this method.

Reader Enquiry No. 106

Embi Tec SmartMark **dsDNA standards** are ladders for sizing dsDNA fragments in polyacrylamide or agarose gels. They consist of dsDNA fragments of precise length and known sequence. Plasmid DNA has been eliminated from the standards and each ladder band has a balanced sequence with 50 per cent G+C content to ensure consistent migration. A 20-bp ladder, a 100-bp ladder and a 200-bp ladder are available. The 20-bp and the 100-bp ladders may be combined to create a 'ruler' from 20 bp to 1,000 bp in 20-bp increments with highlighted bands at 100-bp increments. The 200-bp and 1-kb ladders may be combined to cover the range from 200 to >15 kb, in 200-bp increments with highlighted bands at 1-kb increments.

Reader Enquiry No. 107

Hybaid has developed a new system for **preparing ultrapure DNA** for applications such as transfection of eukaryotic cells or *in vitro* transcription. The Recovery Midi Prep kit is based on the company's spin-prep technology and all purification steps can be performed in a microcentrifuge. According to the company, yields up to 200 µg can be achieved from only 25–30 ml of bacterial culture. Even large plasmids and cosmids up to 100 kb in size can be isolated efficiently. The whole procedure takes less than 40 min and does not require organic extractions or alcohol precipitation steps.

Reader Enquiry No. 108

PCR

Astec Microflow has launched the Microflow Omni **cabinet for use with PCR procedures**. The new cabinet uses laminar airflow technology and has been designed to be highly compact, saving on valuable bench space. The unit is also suitable for other applications where the investigator requires clean air. This unit should provide both operator and prod-

uct protection; it has a clean air flow over the samples and a face velocity between 0.35 m s⁻¹ and 0.4 m s⁻¹. The ultraviolet light has an adjustable timer to ensure maximum operator safety. Furthermore, in order to satisfy safety regulations and COSHH recommendations, the cabinet automatically shuts down the ultraviolet source when it is opened.

Reader Enquiry No. 109

Promega has introduced a new version of its **PCR product cloning vector**, pGEM-T vector. Cloning of PCR products presents unique challenges to the researcher. Many of the DNA polymerases (for example, *Taq* DNA polymerase) used in PCR add a single 3'-deoxyadenosine residue to the ends of PCR products. The pGEM-T vectors have single 3'-T overhangs at the insertion site to complement the overhangs of the PCR product and to facilitate ligation of the DNA into the vector. The new vector combines the benefits of the original vector with easy excision of DNA inserts. The multiple cloning site allows a convenient single restriction digest with *NotI* and *EcoRI*. Both vectors have the promoter



Promega's pGEM-T PCR cloning vector.

sequences for the RNA polymerases T7 and SP6, allowing production of sense and antisense RNA transcripts directly from the vector. The vectors also offer blue/white screening of positive recombinants (white colonies).

Reader Enquiry No. 110

Maxim Biotech now offers a **newsletter** with application information, new product announcements and other news on the company's services and programmes. The premier issue includes the Multiplex PCR (MPCR) optimizer and application kits, improved products for *in situ* hybridization, an RNA/DNA isolation kit and a first strand cDNA synthesis kit. In addition, there are custom services for quantitative competitive PCR, cRNA synthesis by *in vitro* transcription, PCR and MPCR primer/probe design, synthesis and optimization, RNA/DNA isolation and *in situ/in vitro* PCR tissue/cell slides preparation are included.

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READER ENQUIRY NO. 69

Transfection

Stratagene's LipoTaxi **transfection reagent** is a liposome reagent designed to offer simple transfer of DNA into cells. The reagent is stated to combine improved transfection efficacy with the benefits of minimal cellular toxicity. The LipoTaxi reagent yields consistently high transfection efficiencies (greater than eight in ten cells), according to the manufacturer, at least a tenfold increase over calcium phosphate/DEAE-mediated transfection in many lines. It is said to be stable across a wide range of storage temperatures and is easy to use. The company has tested the reagent and states that it is effective in over 20 cell lines and primary cultures. Cell lines where this reagent was demonstrated to be effective as a liposome-mediated transfection reagent include both adherent and suspended cells. Suggested cell lines include 3T3-NIH, H441, MDA-MB-361, A549, HeLa, MDA-MB-453, BHK, JAR, PC-3, CHO, JEG-3, PPC-1, COS-1, Jurkat, Saos-2, DU 145, LNCaP and SK-N-BE(2), DUPRO, lung (primary culture), SK-N-MC, F9, MCG-7 and T47D.

Reader Enquiry No. 112

ExGen 500 from Euromedex is said to be a highly efficient and **non-cytotoxic transfection reagent** that is composed of a mixture of linear ethylenimine polymers. The high cationic charge-density potential of these polymers confers a strong buffering capacity in the endosomal stroma, thus preventing DNA degradation by DNases. This reagent is said to be able to perform high efficiency transfections with submicrogram amounts of plasmids. Its performance is not affected by the presence of fetal calf serum in concentrations up to 30 per cent. Over thirty cell types have been successfully transfected, according to the manufacturer. Two different concentrations are available for *in vivo* and *in vitro* transfection assays.

Reader Enquiry No. 113

Oligonucleotide products

National Biosciences has announced the release of a specification sheet and interactive demonstration disk for Oligo version 5.0, its **oligonucleotide primer analysis software** for the Macintosh. This latest Macintosh version provides 25 new features that make the program more powerful and easier to use, assisting researchers with the selection and management of optimal oligonucleotides for PCR, sequencing, hybridizations, and other applications. All program features are discussed in this four-colour specification sheet and can be reviewed with the demonstration disk. Clicking on any of six stringency settings, ranging from 'very high' to 'very low', automatically pre-sets nine separate search parameters to values

that have been tested and approved by the author of the program. All search-selected primer pairs are listed and compiled in a table along with position number, optimal annealing temperature, PCR product size, and the G+C content of the PCR product. Users can sort by any column and click on any primer pair to show the DNA ampli-

PLK CBP-500					
Optimal Annealing Temperature: 50.4° (max: 58.4°)					
	Position and Length	T _m [°C]	GC [%]	3' ΔG [kcal/mol]	
Product	480	93.9	42.9	-----	
Upper Primer	-3 21	75.3	57.1	-9.9	
Lower Primer	457 21	55.4	28.6	-6.4	
Product T _m - Lower Primer T _m : 28.5					
Primers T _m difference: 19.8					
	Concentration		Excessive difference between product and primer melting temperatures.		
Upper Primer	200.0	nM			
Lower Primer	200.0	nM			
Monovalent Cation	50.0	mM			
Free Mg[2+]	0.7	mM			

Oligo version 5.0 primer analysis software.

cation window. With primer T_m matching, researchers can adjust automatically the length of compatible PCR primer pairs that have disparate T_ms. This adjustment is said to yield cleaner PCR by minimizing false priming through the lower T_m primer. A 5'-GC clamp is said to ensure that all selected oligonucleotides have the desired 5' end necessary for improved priming efficiency. Screening out the stable and very unstable 3' ends is possible with the inclusion of a 3' stability window. With a new algorithm, users can find false priming sites and unique oligonucleotides. Based on a mismatch-sensitive method, priming and false priming conditions are accurately predicted by checking against the active file, multiple files or against files with repeating sequences. The program also offers an oligonucleotide database file that allows downloading and uploading of oligonucleotide sequences and data to and from the Oligo program. The database saves data pertinent to nucleic acid synthesis, and generates a convenient order form

ready to send to the user's synthesis facility. **Reader Enquiry No. 114**

Advanced Biotechnologies now offers heat-stable **streptavidin or oligonucleotide coated, thin-walled tube strips or plates** to fit all leading thermal cyclers with 0.2-ml well heating blocks. The coated tubes can be employed as a pre-amplification separation system, and samples containing target sequences can be directly added to the tubes in lysis buffer. Following binding to biotinylated probes or immobilized oligonucleotides, the DNA or RNA is then amplified using standard protocols. Flat-bottomed spectrophotometer plates are available for identification and quantitation of products using PCR-ELISA technology. The plates are available in packs of five and come free of DNase and RNase and ready for use.

Reader Enquiry No. 115

Genosys Biotechnologies has introduced its OligoPlate, a custom **96-well plate with oligonucleotides covalently attached**. In DNA-based assays, ELISA-type microplate formats are widely used. Moreover, assays using 'capture probes' on microwell surfaces have the advantage of being processed easily in a high-throughput manner, thus reducing labour-intensive steps. Plates can be tailored for any target capture and enable batch analysis of amplified DNA products. These microplate assays can be performed with non-isotopic detection systems (colorimetric, chemiluminescent or fluorescent) and have the potential to be completely automated. For capture assays, Genosys maximizes capture efficiency by attaching the probe to the microwell through a single covalent bond at one end of the probe. The capture probe is attached directly to the plate surface through either a 5'- or 3'-amino modification, leaving the entire length of the probe available for hybridization and resulting in improved signal-to-noise ratios and greater well-to-well reproducibility. The company

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PRODUCT REVIEW

also offers plates coated with a 'universal' capture probe and states that it can synthesize user-specified, target-capture oligonucleotides as a chimaeric probe, including the complement of the universal sequence. Capture assays are performed by a simple hybridization protocol.

Reader Enquiry No. 116

TIB Molbiol offers **custom oligonucleotide synthesis** with a broad range of possible modifications. Multiple fluorescently labelled probes for fluorescence quench applications, for example 5'-3' endonuclease assay (Taqman, Perkin-Elmer), are also now available. One of the company's new services is the synthesis of very long oligonucleotides on polymer film surfaces. The sequences are covalently linked to the polymer film. The products are high quality PCR templates for aptamer generation (SELEX process) and can be used for multiple PCR amplification rounds. The polymer film with the template can easily be removed after the PCR reaction without the need of enzymes, extraction or magnetic separation.

Reader Enquiry No. 117

Gel systems

BlueLine is a new product line for **gel electrophoresis** now available from Serva. The series comprises instruments and accessories for all major electrophoretic applications, including DNA sequencing, DNA agarose submarine, vertical slab-gel electrophoresis of proteins and nucleic acids, horizontal isoelectric focusing and electrophoresis. BlueLine equipment features a modular concept whereby units are compatible with each other. Designed to be easy to use, the BlueVertical vertical slab-gel system has a specially designed casting adapter that allows two gels to be cast in the same core unit as they are run in the electrophoresis tank. It is available either in the mini format (8 cm × 10 cm) to fit pre-cast cassette gels or in the standard format (14 cm × 16 cm). The casting and running stand that comes with the DNA sequencing unit, BlueSeq, is designed to facilitate casting and handling of the sequencing polyacrylamide gels, providing the user with savings in time and effort. The BlueHorizon flatbed chamber uses a cooling plate to suit all horizontal applications (IEF, SDS-PAGE, DNA-electrophoresis) up to a gel format of 245 mm × 125 mm; dual- and triple-electrode techniques are applicable. BluePower programmable power supplies are designed to fit a variety of applications and offer voltages of 200 V, 500 V and 3,000 V.

Reader Enquiry No. 118

Bio-Rad has introduced a fast means of **drying electrophoresis gels** that does not require the use of an expensive vacuum pump. The Gel Air system is a custom

designed drying oven, which can accommodate 16 mini-gels at a time and can have them ready for autoradiography or densitometry in one hour. There is no warm-up time or need to wait for a complete drying cycle when adding more gels. All the user has to do is sandwich the gels between two sheets of cellophane, clamp the sandwich in an assembly frame and place within the system. The dryer occupies little more bench space than one 20 cm × 16 cm gel and requires none of the maintenance associated with vacuum pumps.

Reader Enquiry No. 119

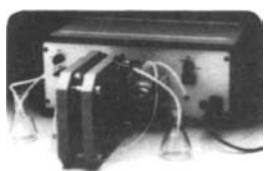
Anachem has launched a range of lightweight, compact **power supplies** to complement the DNA sequencing units and electrophoresis systems being offered. Models PSU-3000/200 and PSU-3000/250P are designed for use with units requiring high voltage, such as the new Anachem Origo V5 and V6 set and Kodak Base Runner sequencing units. The PSU 3000/200 is simple to use and does not require any programming, whereas the versatile PSU 3000/250P is intended for both routine and complex techniques. Programming is easy with the 3000/250P unit, which offers storage memory for 16 one-step and eight multi-step programmes. For the mini- to mid-sized horizontal and vertical gel tanks, Anachem offers the PSU 400/200, which has a capacity to run four gel units at the same time.

Reader Enquiry No. 120

These notes are compiled by Brendan Horton from information provided by the manufacturers. For more details, fill in the reader service card bound inside the journal.

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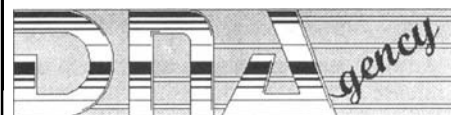
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